PROTEIN PHOSPHATASE ACTIVITY IN THE SALIVARY GLANDS OF THE LONE STAR TICK AMBLYOMMA AMERICANUM (L.)

By

JOHN PHILIP WILLIAMS

Bachelor of Science
in Arts & Sciences
Oklahoma State University
Stillwater, Oklahoma
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Master of Science
Oklahoma State University
Stillwater, Oklahoma
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Thesis Approved:

John R. Anne
Thesis Adviser

Richard C. Essenberg

P. L. Claypool

John A. Bantle

Norman N. Durham
Dean of the Graduate College

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INTRODUCTION

Each part of this thesis is a seperate and complete manuscript to be submitted for publication. Part I has been submitted to Insect Biochemistry. Part II is being submitted to Nature. Each part is presented in the thesis in the format of the journal to which it is submitted.

PART I

PROTEIN PHOSPHATASE ACTIVITY IN TICK SALIVARY GLANDS: CALCIUM DEPENDENT INHIBITION

ABSTRACT

Protein phosphatase activity in tick salivary glands was inhibited by micromolar Ca^{2+} and rabbit skeletal muscle heat stable inhibitor protein and activated by histone H-1. Crude enzyme was separated by anion exchange chromatography at pH 7.2 into two peaks of activity. Both peaks were completely inhibited by micromolar Ca^{2+}. Specific activity increased 50-fold upon dilution. Gel filtration of individual peaks of activity after anion exchange chromatography resulted in a single peak of activity with a M_r of 154 KDa, which was inhibited 98% by micromolar Ca^{2+}. Separation of crude enzyme by anion exchange chromotography at pH 7.5 produced 4 peaks of activity, all of which were inhibited by micromolar Ca^{2+} when phosphorylase a and histone H-1 were substrates but only 50% when exhaustively phosphorylated histone H-2b was the substrate. Further purification by gel filtration gave from each of the four peaks a single peak of activity with a M_r of 154 KDa which was inhibited by micromolar calcium and heat stable inhibitor protein in all cases. Trypsinization of dilute enzyme for 180 min increased activity 4 to 5-fold in the absence of Ca^{2+} and greatly reduced the ability of calcium to inhibit enzyme activity.

Calcium dependant inhibition of protein phosphatase activity demonstrates an important probable function for physiological concentrations of Ca^{2+} in modulating the reversible phosphorylation of proteins in tick salivary glands.

Abbreviations used: MOPS, Morpholinopropane Sulfonic Acid; Tris, (Tris[hydroxymethyl] aminomethane; EDTA, Ethylenediamine tetraacetic acid; EGTA, Ethyleneglycol-bis-(β-aminoethyl ether) N,N, N',N'-Tetraacetic Acid; TCA, Trichloroacetic Acid.

INTRODUCTION

Compelling evidence suggests that dopamine is a neurotransmitter at the neuroeffector junction controlling fluid secretion in ixodid ticks (Kaufman, 1976). Evidence also implicates cAMP as a "second messenger." Exogenous cAMP stimulates fluid secretion by salivary glands in vitro (Sauer et al. 1979) and secretion stimulated by cAMP is augmented by theophylline, an inhibitor of phosphodiesterase (McMullen and Sauer, 1978). Additional evidence of a role for cAMP is the existence of a dopamine sensitive adenylate cyclase in the salivary glands (Schmidt et al., 1981, 1982).

To date, the only known mode of action of cAMP in eucaryotes is to activate protein kinase which catalyzes the phosphorylation of specific proteins. Cyclic AMP dependent protein kinase activity is present in both cytosolic and membrane fractions of tick salivary glands (Mane et al., 1985). In order to modulate cell function via protein phosphorylation a phosphoprotein phosphatase must exist to reverse the reaction catalyzed by protein kinase (Krebs and Beavo, 1979). Reversible phosphorylation of proteins is now recognized as a major mechanism by which cellular metabolism is regulated by neural and hormonal stimuli (Ingebritsen et al. 1980).

Protein phosphatase activity in mammalian tissue has been categorized as Type I or Type II. Type I activity is distinguished from Type II because Type I is inhibited by nanomolar concentrations of two heat stable inhibitor proteins (I-1 and I-2)(Ingebritsen and Cohen, 1983a) and it dephosphorylates the β subunit of phosphorylase kinase. Type II phosphatases dephosphorylate the α subunit of phosphorylase kinase. Type II phosphatases are further subdivided into types 2A, 2B, and 2C, according to their relative substrate specificities, dependance on divalent cations and response to basic proteins.

Protein phosphatase activity in tick salivary glands was indicated in earlier studies by the fact that ^{32}P-labeled phosphate was removed from proteins after adding unlabeled ATP or protein kinase inhibitor to homogenized salivary glands (McSwain et al., 1985).

We report here preliminary data on the characteristics of protein phosphatase activity in tick salivary glands and discuss their probable importance in modulating secretion and other functions in the salivary glands (Sauer and Essenberg, 1984).

MATERIALS AND METHODS

Protein Posphatase and Inhibitor Protein From Rabbit Skeletal Muscle

Type I phosphatase was isolated according to the procedures of Stewart et al. (1981) and heat stable

inhibitors were isolated as described by Aitken et al., (1984).

Preparation of Tick Salivary Gland Homogenates

Salivary glands from feeding adult female lone star ticks Amblyomma americanum, were dissected on ice in 0.1 M MOPS buffer (pH 6.8), containing 20 mM EGTA. Salivary glands were stored in 0.1 M PIPES, (piperazine N,N bis (2-ethane sulfonic acid)) pH 6.8, 20 mM EGTA and 40% glycerol at -10°C before use. EGTA was included to inhibit the high Ca^{2+} - dependent protease activity in tick salivary glands and was the most effective protease inhibitor tested (Mane et al., 1986).

Prior to homogenization, salivary glands were washed three times with 5 volumes of 50 mM PIPES pH 7.0, 1.0 mM dithiothreitol (DTT), 2 mM EGTA, and 5 mM glycerol-2-phosphate. The salivary glands were homogenized in the same buffer with a Tekmar SDT-1810 Tissumizer, centrifuged at 15,000 x g for 5 minutes and the supernatant used as the source of enzyme in assays of crude homogenate.

Assay of Protein Phosphatase Activity

Protein phosphatase activity was assayed at 37°C by measuring the release of ^{32}P from labeled substrates (Ingebritsen and Cohen, 1983b). The incubation medium included 50 mM PIPES buffer pH 7.0, 0.5 mM DTT, 2 mM EGTA, 5 mM glycerol-2-phosphate and 1 mg/ml bovine serum albumin

(BSA). Enzyme (5µl) was preincubated 2 minutes in buffer prior to initiating the reaction by adding labeled phosphoprotein substrates (50µl final volume). The reaction was terminated after 10 min with 200 µl 50% TCA. After 10 min on ice, 200 µl BSA (30 mg/ml) was then added to the mixture. The suspension was vortexed placed on ice for an additional 10 min and centrifuged at 15000 x g for 5 min. A 100 µl aliquot of supernatant was placed in 5 ml BioCount scintillation fluid and radioactivity measured with a Beckman LS-3133T liquid scintillation counter.

To test for possible Ca^{2+} dependent protein phosphatase activity (phosphatase 2B, or calcineurin in mammalian tissue), levels of free calcium were set with a Ca-EGTA buffer using a computer program (Perrin and Sayce, 1967) to find the required concentrations. The amount of free calcium in the incubation medium was estimated based on amounts and the binding constants of all ligands (glycerol-2-phosphate and EGTA) in the incubation medium.

Preparation of ^{32}P Labeled Substrates

Various buffers were used in the preparation of ^{32}P-labeled substrates and during column chromatography: A. 50 mM Tris, pH 7.0, 1 mM EDTA, 25 mM NaF and 14 mM 2-mercapthoethanol. B. 50 mM Tris, pH 7.0, 1 mM EGTA and 50 mM 2-mercaptoethanol. C. 50 mM Tris, pH 7.2, 2 mM EGTA, and 45 mM 2-mercaptoethanol. D. 50 mM Tris, pH 7.5, 2mM

EGTA and 14 mM 2-mercaptoethanol. E. 20 mM MOPS, pH 6.5, 10 mM EGTA and 14 mM 2-mercaptoethanol.

Histone H-1 was prepared from calf thymus (Jones, 1964) and phosphorylase b, phosphorylase kinase and cAMP dependent protein kinase were obtained from Sigma Chemical Co. (St. Louis). [γ- ^{32}P]-ATP (6000 µCi/mmole) was synthesized according to Walseth and Johnson (1979).

Histone H-1 was phosphorylated at room temperature for 2 hours in 50 mM PIPES, pH 7.0 containing histone H-1 (3mg/ml), 0.4 mM EDTA, 0.2 mM EGTA, 2 mM magnesium acetate, 25 mM NaF, 10 mM glycerol-2-phosphate, 0.2 mM [γ-^{32}P]-ATP, 10 µM cAMP and 0.15 mg/ml cAMP dependent protein kinase in a final volume of 5 ml. The reaction was stopped with 0.1 volume of 50% TCA. The reaction mixture was placed on ice for 30 min and centrifuged at 15,000 x g for 10 min. The pellet was resuspended in 5 ml of buffer A and dialyzed against 800 volumes of buffer A for 48 hrs with 4 changes of buffer.

To prepare phosphorylase a (Antoniw et al., 1977), phosphorylase b (2.5 mg/ml) was incubated for 2 hr at room temperature in 50 mM Tris (pH 7.0) with 0.03 mg/ml phosphorylase kinase, 0.2 mM [γ-^{32}P]-ATP, 10 mM glycerol-2-phosphate and 10 mM magnesium acetate in a final volume of 5 ml. The reaction was terminated with 1 volume of 90% saturated ammonium sulfate (pH 7.0) and placed on ice for 30 min before centrifugation at 15000 x g for 10 min. The 15000 x g pellet was washed with buffer B containing 45%

ammonium sulfate and again centrifuged at 15000 x g for 10 min. The second 15000 x g pellet was resuspended in 2.5 ml of buffer B and dialyzed against 1600 volumes of buffer B for 48 hrs with 4 changes of buffer. The crystals of phosphorylase a formed during dialysis were collected after 10 min of 10,000 x g centrifugation and resuspended in buffer B containing 250 mM NaCl at a final concentration of 5 mg/ml.

DEAE Sephacel Chromatography

Salivary glands from 750 feeding adult female ticks were dissected as described previously and homogenized in buffer C. After centrifugation at 40,000 x g for 20 min, the supernatant was filtered over glass wool and chromatographed on a DEAE Sephacel column (1.6 x 12 cm) equilibrated with 150 ml buffer C. The column was washed with buffer C and bound proteins were eluted with a linear gradient of 0-0.5 M NaCl in buffer C. Phosphatase activity was not detected in unbound fractions. Fractions (2.8 ml) were assayed for phosphatase activity as described previously.

A second DEAE Sephacel column was equilibrated with buffer D. Crude homogenate was prepared as before but in buffer D, loaded on the column, and proteins eluted (2.6 ml fractions) with a linear gradient of 0-0.4 M NaCl in buffer D and fractions assayed for phosphatase activity as previously described.

Sephacryl S-300 Gel Filtration

Peak fractions from the DEAE Sephacel columns were pooled separately and concentrated on Centricon C-10 microconcentrator tubes. Each sample was dialyzed in equilibration buffer (20 mM MOPS, pH 6.5, 10mM EGTA and 0.1% 2-mercaptoethanol) and placed on a Sephacryl S-300 column (0.5 x 80 cm). Fractions (2.4 or 2.6 ml) were collected and assayed as previously described.

Enzyme Trypsinization

Two ml of enzyme from Peak I following anion exchange chromatography at pH 7.2 were incubated at room temperature with 0.1 µg/µl trypsin for up to 3 hr. At various times 200 µl aliquots were withdrawn and added to a microcentrifuge tube containing 50 µg soybean trypsin inhibitor, vortexed and placed on ice. Enzyme activity in the presence and absence of Ca^{2+} was assayed as described previously.

RESULTS

Enzyme Specific Activity

Activity of the enzyme was not a linear function of enzyme concentration. As shown in Fig. 1, at two different concentrations, the specific activity was quite different (8 pmol ^{32}P released/min/µg protein at 0.06 µg protein/µl and 0.16 pmol ^{32}P/min/µg protein at 0.6 µg/µl), but in each case, the time course was linear. Gel filtration

demonstrated a M_r of 154 KDa for concentrated enzyme and 74 KDa for the diluted enzyme (Fig. 2).

Modulation of Tick Salivary Gland and Rabbit Skeletal Muscle Protein Phosphatase Activity by Rabbit Skeletal Muscle Inhibitor Protein

Increasing concentrations of heat stable inhibitor proteins (1.24 µg protein/µl) from rabbit skeletal muscle inhibited crude protein phosphatase activity by 41% in tick salivary glands (suggesting type 1 phosphatase activity) with phosphorylase a as substrate (Fig 3). In comparison, protein phosphatase activity from rabbit skeletal muscle was inhibited by approximately 65%. Both enzymes were inhibited maximally by the same concentration of inhibitor protein.

Effect of Histone H-1 on Tick Phosphatase Activity

Protein phosphatase 2A in vertebrate tissue can be activated by basic proteins such as histone H-1 (Ingebritsen and Cohen, 1983b). To determine if tick salivary gland protein phosphatase activity could be activated by a basic protein, increasing concentrations of unlabeled histone were added to assays with phosphorylase a serving as substrate. Enzyme activity of crude tissue was unaffected (data not shown) but enzyme activity from Peak I following anion exchange chromatography at pH 7.5 was increased 2-fold by inclusion of 8 µM histone H-1 (Fig. 4). Activity declined slightly at concentrations between 15-20 µM histone H-1.

Effect of EGTA on Phosphatase Activity

Protein phosphatase activity was assayed in crude preparations with increasing concentrations of EGTA in attempts to determine if Ca^{2+} dependent activity was present in tick salivary glands. Activity in the presence of 5 mM Mn^{2+}, a divalent cation required to stabilize calcineurin activity in mammalian tissue (Stewart et al., 1983), but without EGTA was two-fold higher than in the absence of Mn^{2+}, and was inhibited approximately 60% by 4 mM and above EGTA (Fig. 5). In the absence of Mn^{2+}, there was a very slight decline in activity. Some EGTA-inhibited activity was restored by adding Ca^{2+} and calmodulin (data not shown), but results were inconsistent. The level of free Ca^{2+} required to stimulate mammalian calcineurin activity is low (< 1μM). Therefore levels of free Ca^{2+} were more carefully regulated with a Ca^{2+}-EGTA buffer system. In the presence of between 1.2 and 1.7 mM Mn^{2+} little effect of calcium was observed (Fig 6). In the absence of Mn^{2+}, phosphatase activity was increased 11-fold at low concentrations of Ca^{2+}. This increased activity was inhibited at higher Ca^{2+} concentrations, activity with and without Mn^{2+} being almost the same at 1 μM Ca^{2+} (Fig 6).

DEAE Sephacel Chromatography

To resolve possible forms of protein phosphatase activity, and to test further the effects of Ca^{2+}, salivary gland supernatant from 750 feeding female ticks was

chromatographed on a DEAE Sephacel column equilibrated in buffer C at pH 7.2. In these experiments, fractions were assayed with and without 1 μM Ca^{2+} using labeled histone H-2b (0.75 mol ^{32}P incorporated/mol H-2b) as substrate. Major and minor peaks (Peak I and Peak II) of activity were obtained without calcium (Fig 7). Phosphatase activity in all fractions was almost completely inhibited by 1 μM Ca^{2+}. Peak I and Peak II activities from anion exchange chromatography were then pooled separately, concentrated, dialyzed against buffer E and chromatographed on Sephacryl S-300. Both Peak I and Peak II activity eluted with M_r of 154 KDa and were almost completely inhibited by 1 μM Ca^{2+} (Data not shown). To determine if pH was a factor affecting resolution of forms of protein phosphatase, supernatant (40,000 x g) from glands of 1000 feeding female ticks was chromatographed on a second DEAE Sephacel column at pH 7.5. Fractions were assayed with phosphorylase a as substrate alone and in the presence of 10 μl of heat-stable inhibitor or 5 μM histone H-1. All fractions showing activity against phosphorylase a were stimulated by histone and inhibited by the inhibitor (Fig. 8a). These fractions were also assayed with labeled histone H-1 (0.8 mol ^{32}P incorporated/mol H-1) as substrate and the effects of 1 μM Ca^{2+} and 200 μM TFP were determined. All fractions with protein phosphatase activity were inhibited by micromolar calcium (Fig 8b). Trifluoperazine and Ca^{2+} had the same effect as calcium alone (data not shown). With exhaustively

phosphorylated histone H-2b as substrate (1.5 mol ^{32}P incorporated/mol H-2b), 1 μM Ca^{2+} inhibited phosphatase activity in these fractions approximately 50% and the addition of 200 μM TFP in the presence of 1 μM Ca^{2+} caused an additional 25% inhibition (Data not shown).

In all, at least four peaks of phosphatase activity were resolved using two phosphoprotein substrates and anion exchange chromatography at pH 7.5 (Fig. 8). Peak I and II were apparent with both substrates while Peak III was only apparent using phosphorylase a and Peak IV with histone H-1. All peaks were pooled separately, concentrated, dialyzed against Buffer E and further chromatographed on Sephacryl S-300. All four peaks resulted in a single peak of activity with a M_r of 154 KDa (data not shown). As before, all fractions from each peak were partly inhibited by heat stable inhibitor protein and completely inhibited by micromolar Ca^{2+}.

Effect of Trypsinization

Enzyme from Peak I of the pH 7.2 DEAE Sephacel column was subjected to 3 hrs trypsinization in an attempt to determine if a factor responsible for mediating the calcium-dependent inhibition could be removed or altered by proteolysis. Phosphatase activity was assayed at various times with and without 1 μM Ca^{2+}. Enzyme activity in the absence of Ca^{2+} with histone H-1 as substrate was inhibited approximately 50% after the first 10 min of digestion with

trypsin and then slightly more for the next 110 min (Fig 9a). After 120 min digestion with trypsin, enzyme was still completely inhibited by 1 μM Ca^{2+}. However, activity without Ca^{2+} increased 4.5 fold between 120 and 180 min digestion with the protease. At the same time activity in the presence of Ca^{2+} was greatly increased but was still less than without Ca^{2+}. Similar results were obtained using phosphorylase a as substrate (Fig 9b) except the initial decrease in phosphatase activity after 10 min pre-incubation with trypsin was not observed. The enzyme was not rechromatographed to see if trypsinization caused formation of additional molecular forms of the enzyme.

DISCUSSION

Tick salivary gland protein phosphatase activity appears to exist as a dimer with a M_r of 154 KDa in concentrated preparations and smaller possibly monomeric subunits with a M_r of 74 KDa after dilution. Both forms are affected by the various factors which affect protein phosphatase activity in vertebrate tissues. Furthermore, dilution causes a 50-fold activation of the enzyme. The physiological significance of these results are not clear.

Crude protein phosphatase activity in tick salivary glands was inhibited by heat stable inhibitor protein but not to the same extent as protein phosphatase 1 from rabbit skeletal muscle. Because the inhibitor preparation was not phosphorylated by cAMP dependent protein kinase (Ingebritsen

and Cohen, 1983a), most of the inhibitor protein was probably I-2 although we cannot exclude the possibility of contamination by endogenously phosphorylated I-1 isolated during purification procedures. Further, since rabbit skeletal muscle phosphatase I was not purified to homogeneity, incomplete inhibition by inhibitor proteins may be due to contamination by Type II phosphatases.

Both phosphatases 1 and 2A from vertebrate tissue readily dephosphorylate phosphorylase a (Ingebritsen & Cohen, 1983b) and this molecule was a good substrate for tick protein phosphatase. Phosphatase I dephosphorylates the β-subunit of phosphorylase kinase and Type II dephosphorylates the α-subunit of this enzyme. Thus it was surprising that phosphorylase kinase was a poor substrate for crude salivary gland protein phosphatase. Vertebrate phosphatase 2A is activated (about 2-fold) by basic proteins such as histone H-1 and protamine, which conversely are potent inhibitors of protein phosphatase-1. Salivary gland protein phosphatase was activated by histone H-1 only after partial purification on anion exchange chromatography. These results suggest that both protein phosphatase 1 and 2A-like activity are present in tick salivary glands but these enzymes appear to be different from vertebrate forms based on substrate specificity.

Phosphatase 2B, or calcineurin, is a Ca^{2+} calmodulin binding protein that was first noted as an inhibitor of calmodulin-dependent phosphodiesterase (Klee and Krinks,

1978). Stewart et al. (1983) showed that calcineurin was a Ca^{2+}, calmodulin dependent protein phosphatase. Calcineurin is stimulated almost 2 fold by 1 μM Ca^{2+} (Tallant and Cheung, 1984). In the presence of 1 μM Ca^{2+} and 3 nM calmodulin activity is stimulated 10-15 fold (Wallace et. al. 1978). Experiments with increasing concentrations of EGTA in the presence of 5 mM Mn^{2+} failed to demonstrate Ca^{2+} dependent phosphatase activity. Inhibition of phosphatase activity was probably due to chelation of Mn^{2+} since similar inhibition was not noted in the absence of Mn^{2+} (Fig. 5). The significance of the stimulatory affect of 5 mM Mn^{2+} is unclear since Mn^{2+} concentrations between 1.2 and 1.7 mM were inhibitory. These results are also unusual because the physiological concentration of Mn^{2+} in most cells is low. In the absence of Mn^{2+}, enzyme activity was 11-fold higher at very low Ca^{2+} concentrations (Fig. 6), but was inhibited by 0.1 and 1 μM free Ca^{2+}. It is interesting that 1-2 mM Mn^{2+}, in the presence of Ca^{2+}, is as inhibitory as Mn^{2+} alone (Fig. 6). It is possible that Mn^{2+} may substitute for Ca^{2+} since higher concentrations of Ca^{2+} do not inhibit the enzyme to a greater extent. High levels of Mg^{2+} block the Ca^{2+} inhibitory effect (unpublished observation), but Mg^{2+} by itself has no effect on enzyme activity. Magnesium is a requirement for 2C enzyme activity in vertebrate tissue (Ingebritsen and Cohen, 1983b).

Inhibition of phosphatase activity by TFP is interesting when contrasted with inhibition by Ca^{2+} alone.

TFP was more inhibitory than Ca^{2+} only if the substrate was exhaustively phosphorylated (histone H-2b is phosphorylated on two serine residues). Trifluoperazine binds specifically to calmodulin at concentrations less than 0.1 mM (Levin and Weiss, 1977) and only to the Ca^{2+} calmodulin complex. Inhibition by TFP at these levels usually is an indication of a process activated by Ca^{2+} acting through calmodulin. Enzyme activity with partially phosphorylated histone H-2b (0.75 mol ^{32}P incorporated/mol H-2b) was completely inhibited by micromolar Ca^{2+} while activity was inhibited 50% with exhaustively phosphorylated histone H-2b as substrate. Complete inhibition was also apparent when labelled histone H-1, which is phosphorylated on a single serine residue, was used as substrate.

Anion exchange chromatography on DEAE Sephacel resulted in 2 (pH 7.2) or 4 (pH 7.5) peaks of phosphatase activity. The 2 peaks of activity resolved at pH 7.2 were further purified with Sephacryl S-300 gel filtration. Both peaks eluted with a M_r of 154 KDa. Activity from both peaks were partially inhibited by rabbit skeletal muscle heat stable inhibitor protein and completely inhibited by micromolar Ca^{2+}. Activity within all 4 peaks from the second DEAE column (pH 7.5) eluted as single peaks of activity on gel filtration with maximum activity at 154 KDa. Phosphatase activity was again partially inhibited by the heat stable inhibitor protein and completely inhibited by micromolar Ca^{2+}.

Enzyme activity from crude salivary gland homogenates was not inhibited to the same extent by 1 μM Ca^{2+} as partially purified fractions suggesting the presence of an additional labile or separable modulator of Ca^{2+}-dependent inhibition. Whether such a modulator is removed by limited proteolysis, separated or dissociated from the native enzyme under conditions of column chromatography is not known. The modulator is not likely a Ca^{2+}-binding protein because excess Ca^{2+} does not increase inhibition of enzyme activity in crude tissue (unpublished observation).

Protein phosphatase activity from fraction 44 of Peak I following anion exchange chromatography was subjected to trypsinization for up to 3 hrs. As before, 1 μM Ca^{2+} inhibited enzyme activity even after 2 hours of trypsinization. Both Ca^{2+}-inhibited and non-inhibited activity were increased after an additional hour of incubation with the protease. However, the ability of Ca^{2+} to inhibit activity was greatly reduced after 3 hrs of trypsinization. The cause and significance of activation of phosphatase activity following extensive trypsinization are not known. However, protein phosphatase activity can be activated by limited proteolysis in other tissues (Yang, 1985). All activity detected in this study was inhibited by μM Ca^{2+} and by 1-2 mM of Mn^{2+}. Ca^{2+} inhibition was antagonized by high concentrations of Mg^{2+}. The factor mediating the Ca^{2+}-dependent inhibition may be calmodulin or

a calmodulin-like molecule and appears to be an integral subunit of the enzyme.

We hypothesize that the phosphorylation of proteins caused by increases in salivary gland cAMP would be enhanced by simultaneous inhibition of protein phosphatase activity. Increasing the cytosolic concentration of calcium may result in the inhibition of protein phosphatase activity thereby amplifying the effect of cAMP. In this connection it seems significant that dopamine-stimulated salivary fluid secretion (in vitro) by ixodid ticks is reduced by deletion or lowering the concentration of exogenous calcium in the bathing medium (Needham and Sauer, 1979).

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Figure 1. 100 µl of both a crude enzyme ( ) preparation (6 µg protein/µl) and a 10-fold dilution ( ) were assayed in a final volume of 1 ml. 50 µl aliquots were removed at 1,3,5,10,15 and 20 min and assayed for phosphatase activity. Data are presented as phosphate release as a function of the product of enzyme concentration (µg protein) and the time of incubation.

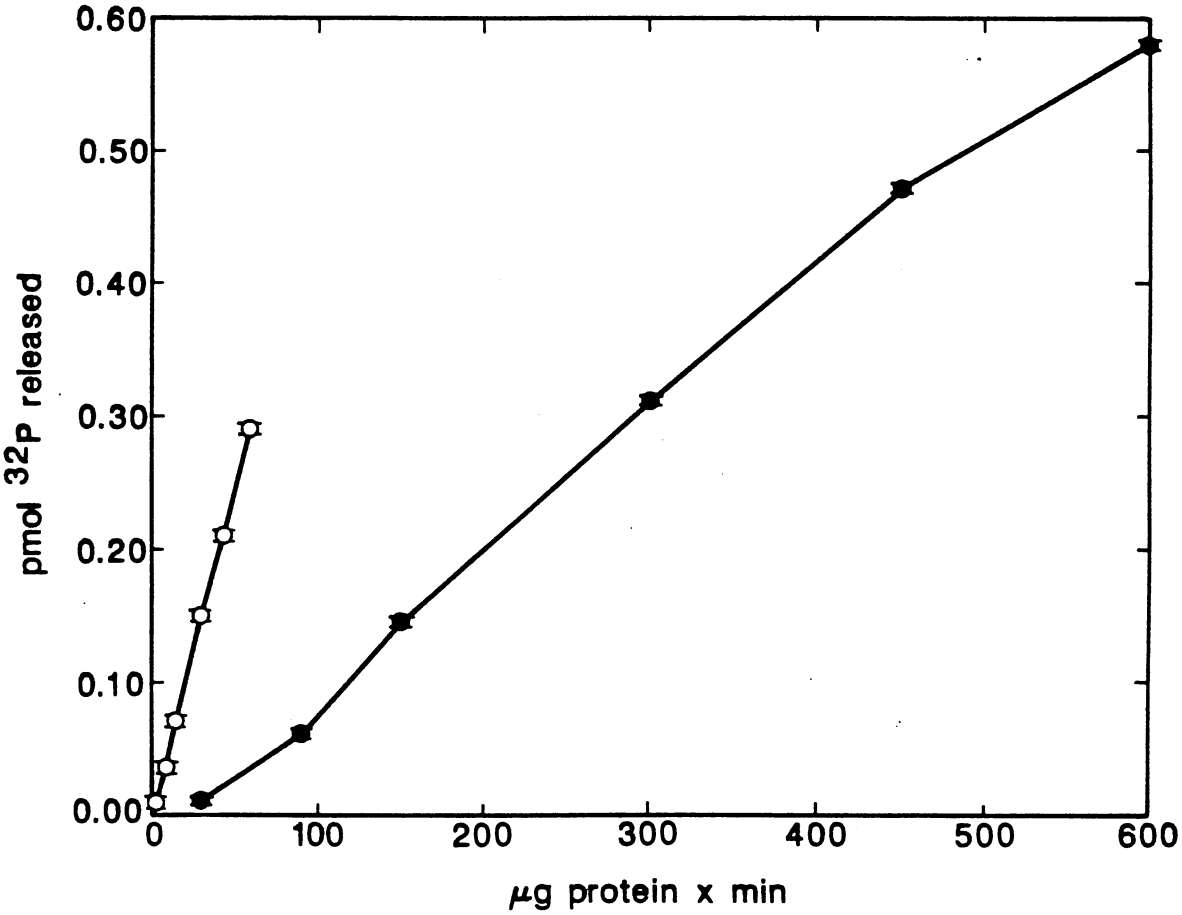

0.60
0.50
0.40
0.30
0.20
0.10
0.00
pmol 32P released
0
100
200
300
400
500
600
μg protein x min

Figure 2. Sephacryl S-300 gel filtration of concentrated ( ) (6 µg/µl) and 7-fold diluted ( ), (0.9 µg/µl) enzyme preparations. Fractions were assayed for phosphatase activity using phosphorylase a as substrate.

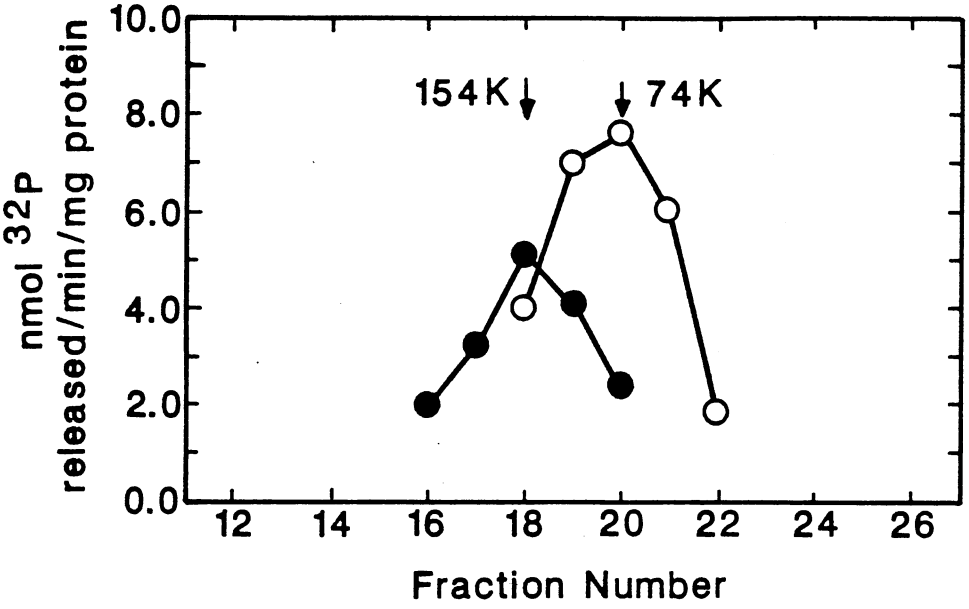

10.0
8.0
6.0
4.0
2.0
0.0
nmol 32P released/min/mg protein
154K
74K
12
14
16
18
20
22
24
26
Fraction Number

Figure 3. Inhibition of tick salivary gland ( ) and rabbit skeletal muscle ( ) protein phosphatase by the heat stable inhibitor protein from rabbit skeletal muscle. Crude supernatant (15,000 x g) from tick salivary glands and partially purified enzyme from rabbit skeletal muscle were assayed with phosphorylase a as substrate at increasing concentrations of inhibitor protein (1.24 µg protein/µl).

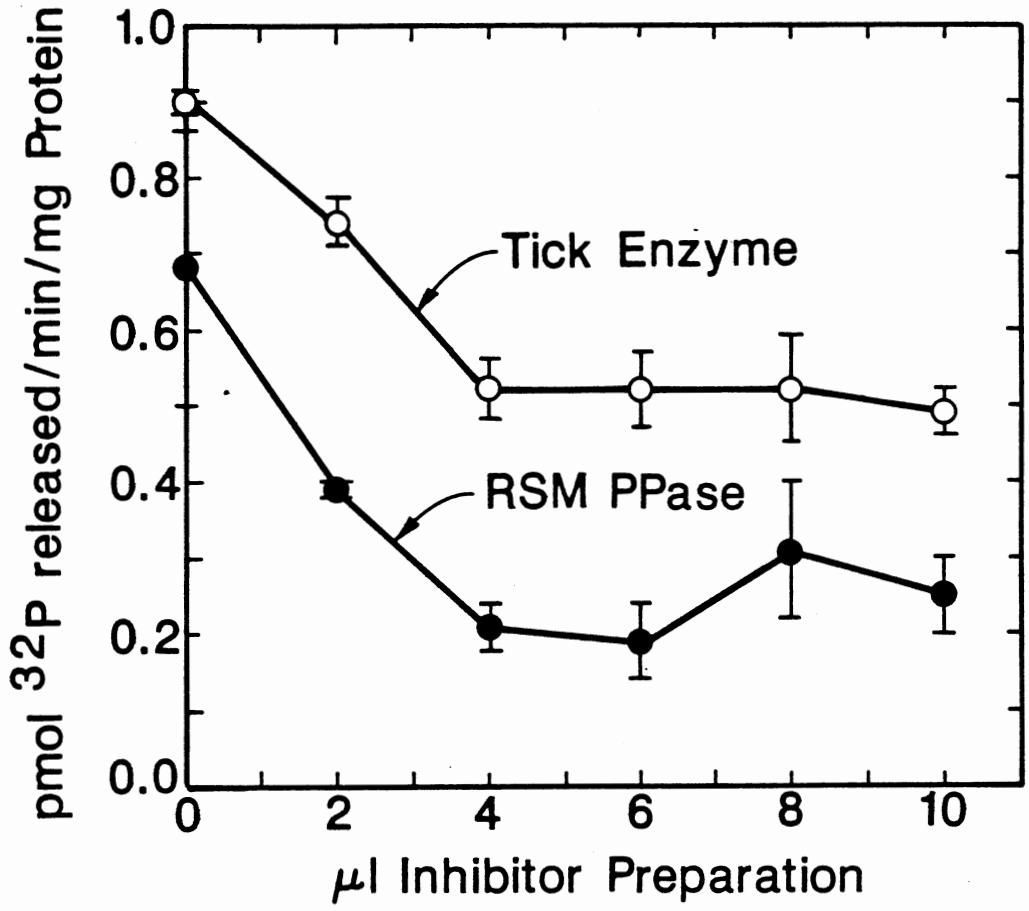

pmol 32P released/min/mg Protein
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
μl Inhibitor Preparation
Tick Enzyme
RSM PPase

Figure 4. Effect of increasing concentration of unlabeled histone H-1 on partially purified protein phosphatase activity. Enzyme that had previously been subjected to anion exchange chromatography was in the presence of increasing concentrations of histone H-1 using phosphorylase a as substrate.

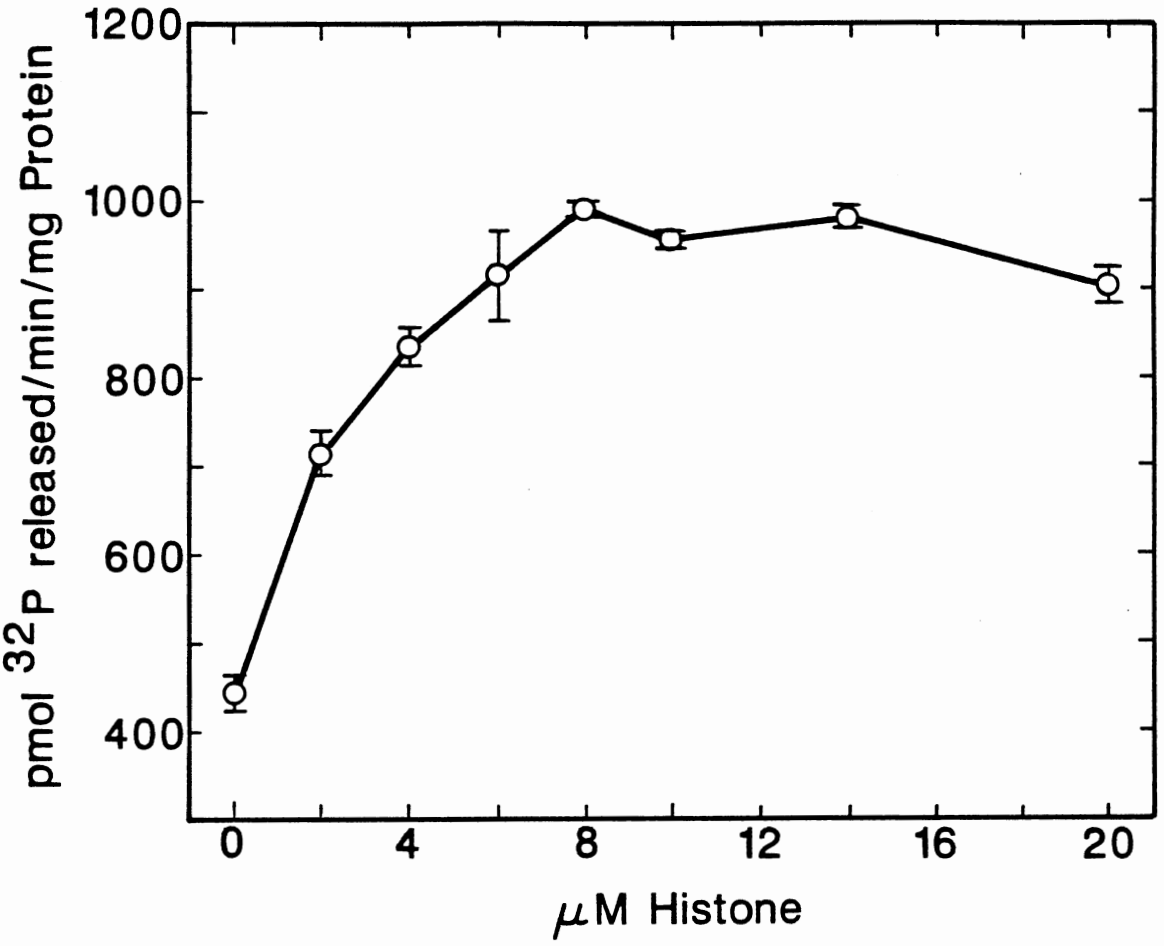

1200
1000
800
600
400
pmol ^{32}P released/min/mg Protein
0
4
8
12
16
20
μM Histone

Figure 5. Effect of increasing concentration of EGTA on protein phosphatase activity in salivary gland supernatant (15,000 x g). Enzyme activity was assayed in the absence ( ) or presence ( ) of 5 mM $MnCl_2$ and 0 to 20 mM EGTA.

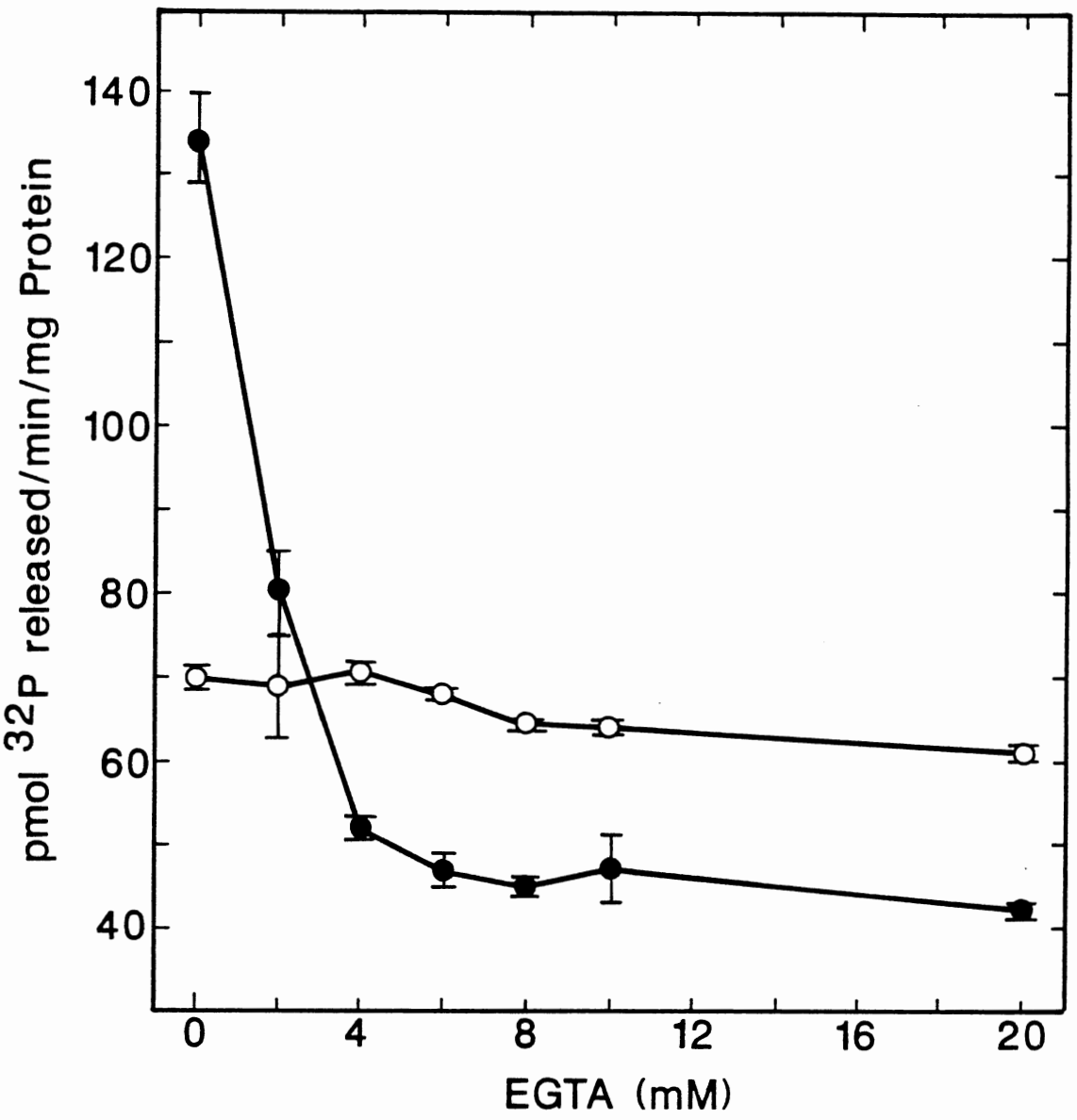

140
120
100
80
60
40
pmol ^{32}P released/min/mg Protein
0
4
8
12
16
20
EGTA (mM)

Figure 6. Effect of Ca^{2+} on crude salivary gland supernatant (15,000 x g) protein phosphatase activity. A Ca^{2+}-EGTA buffer was used to control the level of free Ca^{2+}. Activity was assayed in the presence ( ) or absence ( ) of 1.2 to 1.7 mM $MnCl_2$ with varying concentrations of Ca^{2+} and phosphorylase a as substrate.

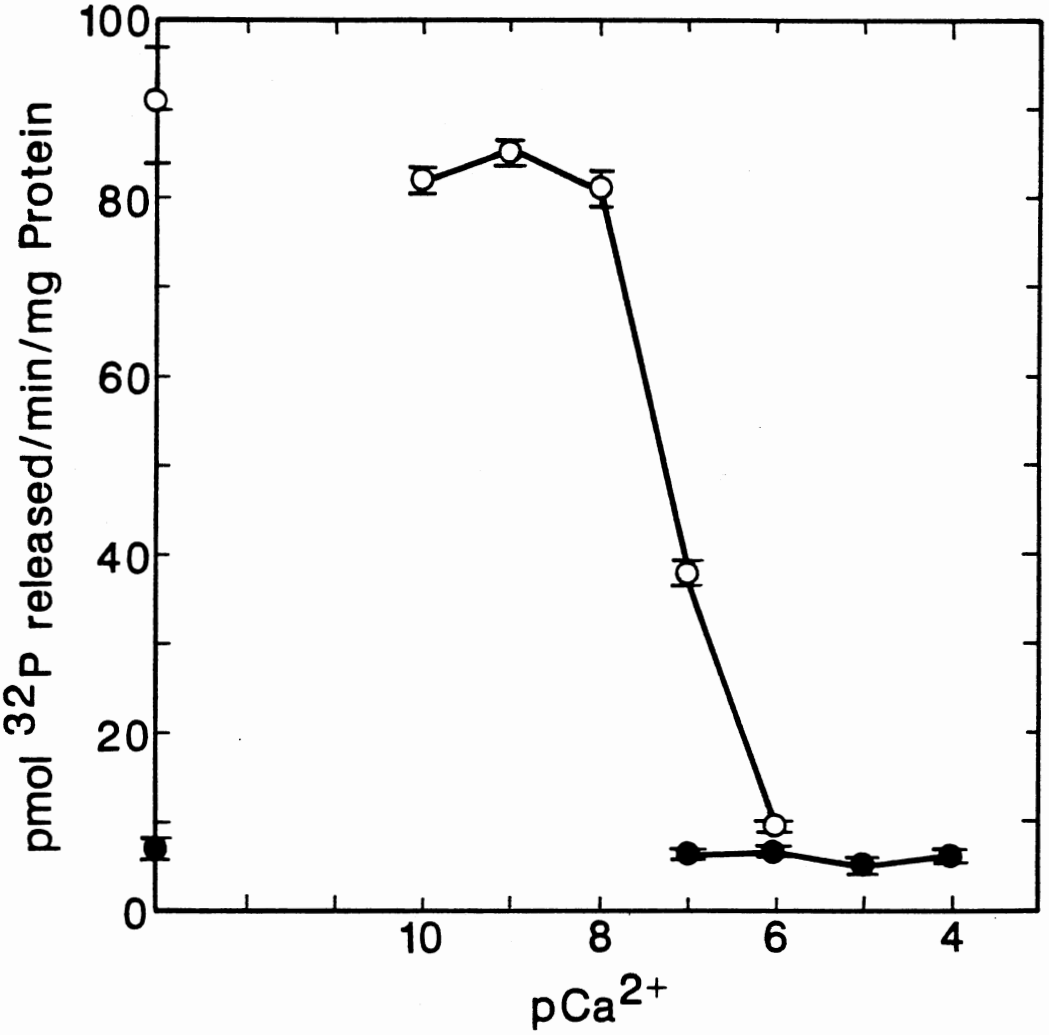

100
80
60
40
20
0
pmol ^{32}P released/min/mg Protein
10
8
6
4
pCa^{2+}

Figure 7. DEAE Sephacel anion exchange chromatography at pH 7.2 of salivary gland homogenate from feeding female lone star ticks. 2.6 ml fractions were collected with a linear gradient of 0 - 0.5 M NaCl. Phosphatase activity was assayed with partially phosphorylated histone H-2b as substrate in the absence ( ) or presence ( ) of 1μM Ca^{2+}.

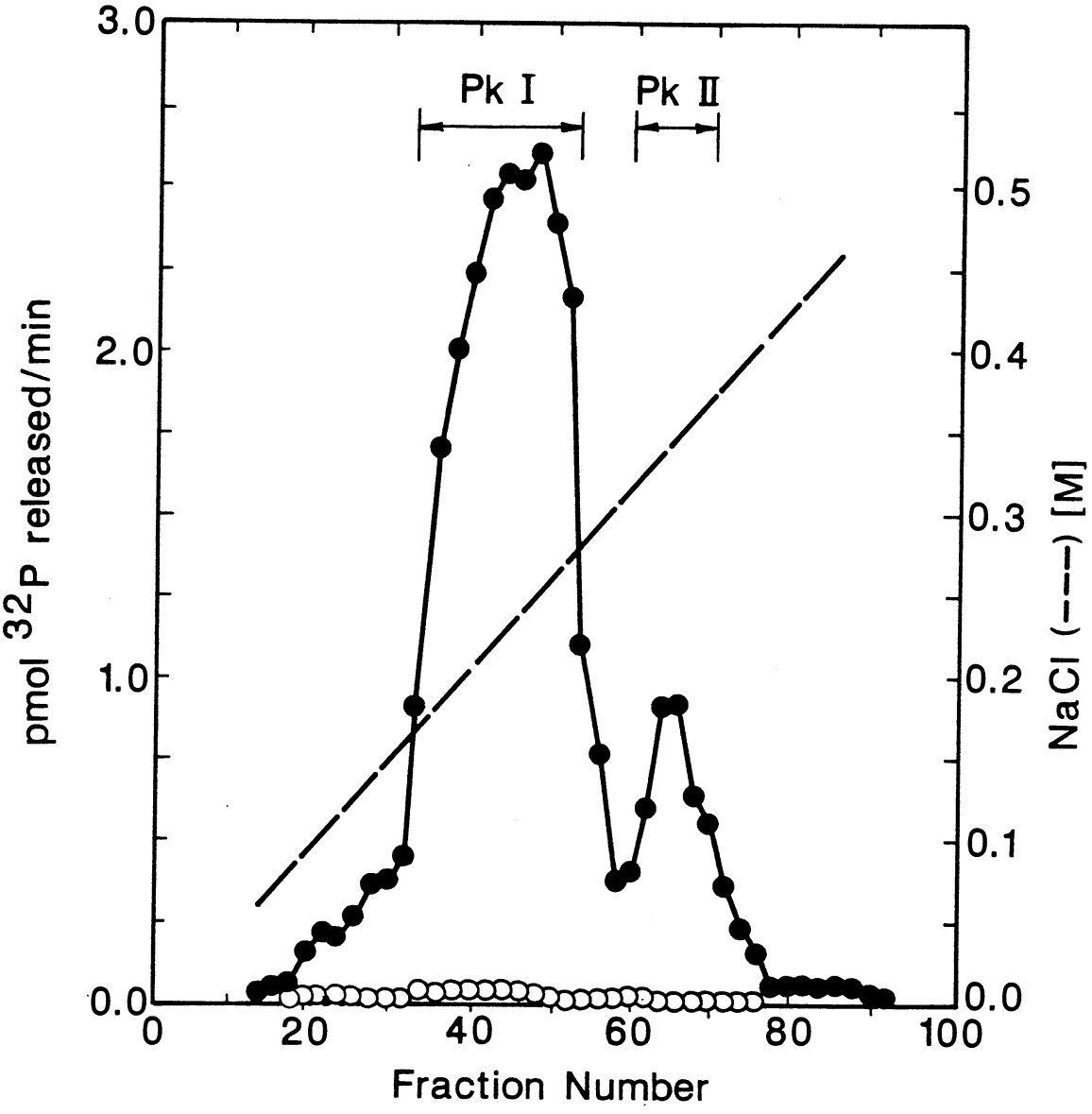

3.0
2.0
1.0
0.0
pmol ^{32}P released/min
Pk I
Pk II
0.5
0.4
0.3
0.2
0.1
0.0
NaCl (----) [M]
0
20
40
60
80
100
Fraction Number

Figure 8. DEAE Sephacel anion exchange chromatography at pH 7.5 of salivary gland homogenate from feeding female lone star ticks. 2.6 mL fractions were collected with a linear gradient of 0 - 0.4 M NaCl. a) Fractions were assayed for phosphatase activity without additional factors ( ) in the presence of 5µM unlabeled histone H-1 ( ) or 10µl (1.24 µg/µl) of rabbit skeletal muscle inhibitor ( ) using phosphorylase a as substrate. b) Fractions were assayed for protein phosphatase activity in the absence ( ) or presence ( ) of 1 µM Ca^{2+} using histone H-1 as substrate.

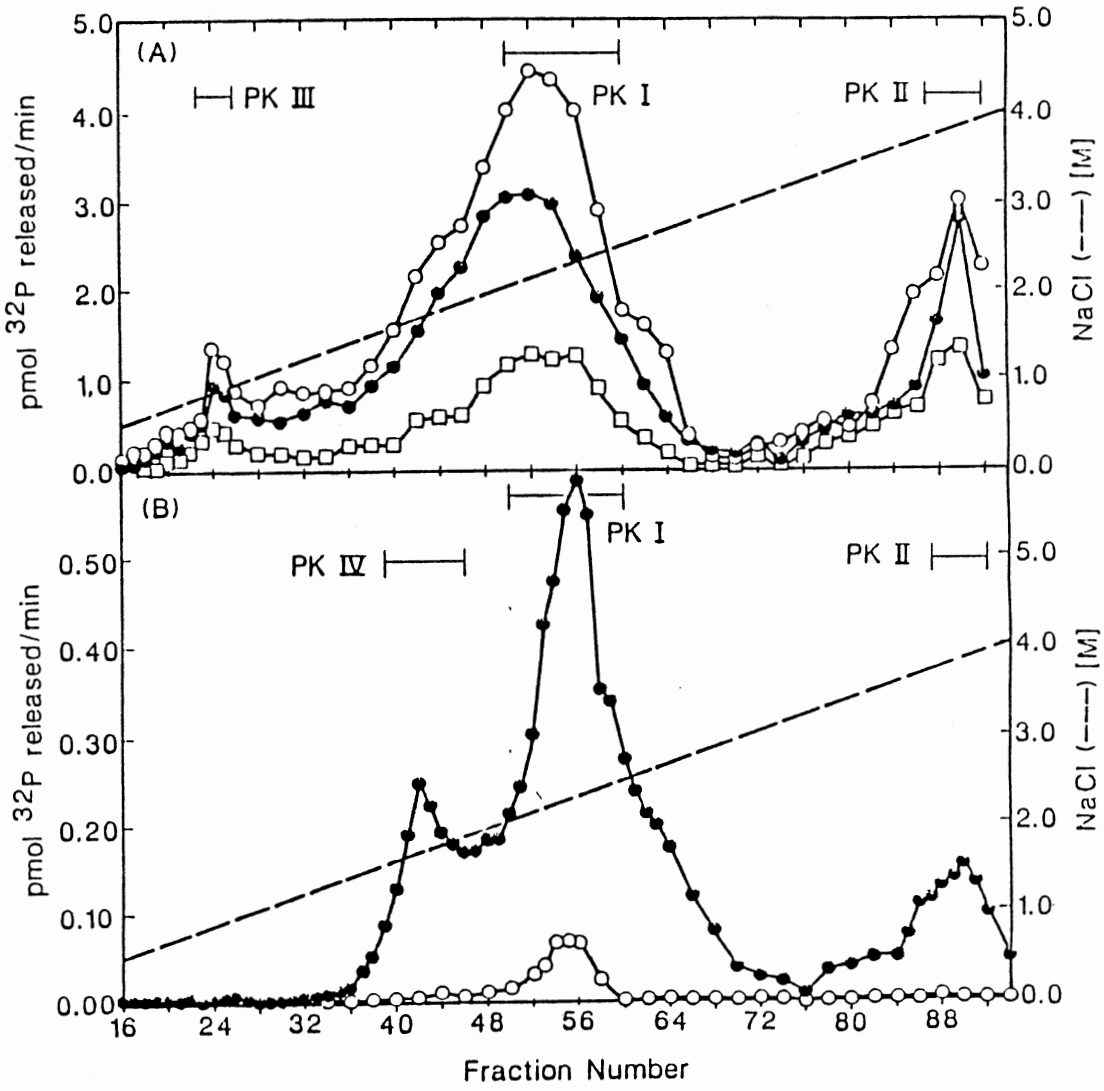
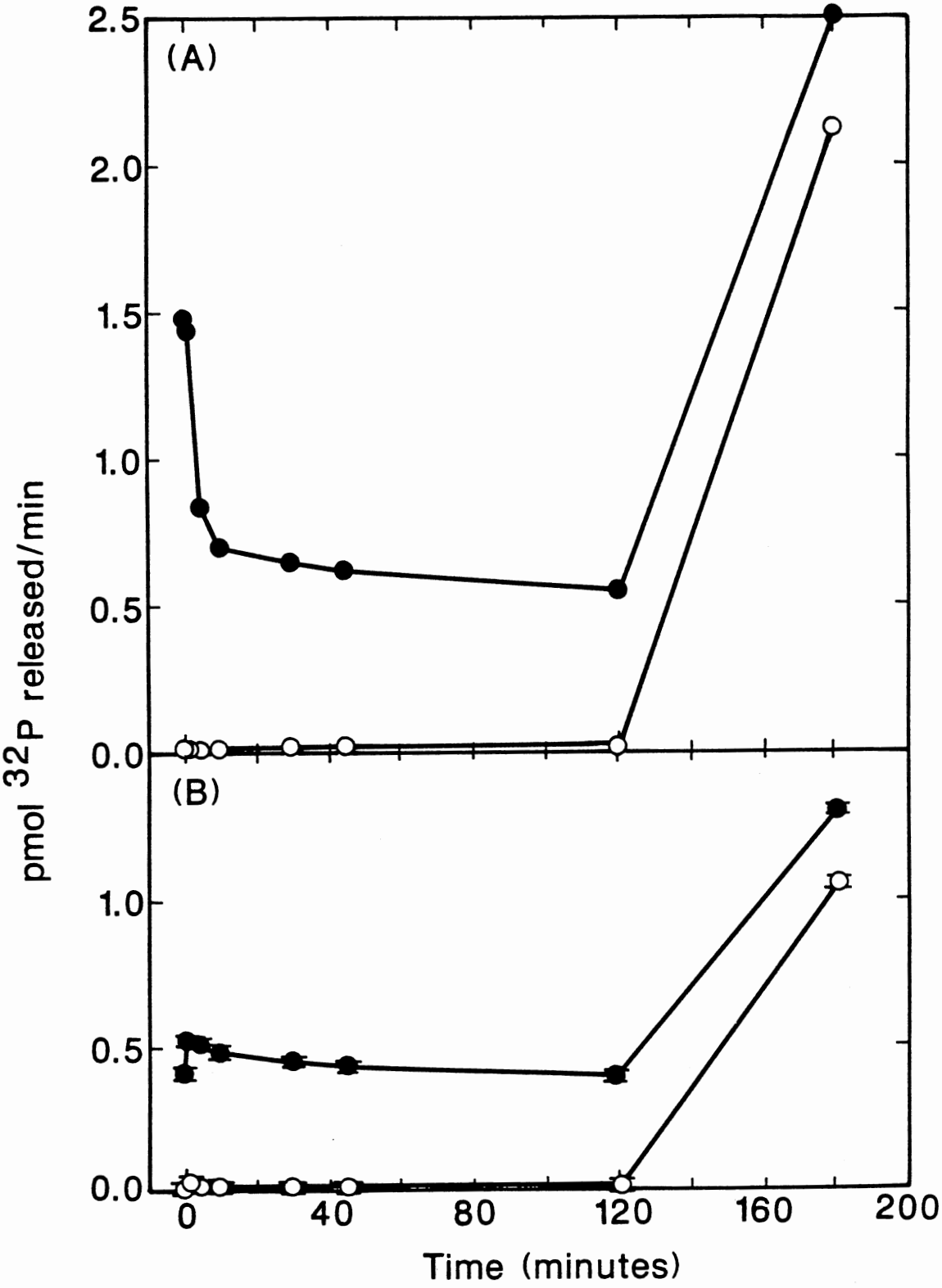

(A)
PK III
PK I
PK II
pmol ^{32}P released/min
NaCl (——) [M]
(B)
PK IV
PK I
PK II
Fraction Number

Figure 9. Effect of trypsinization on protein phosphatase activity. Enzyme (2 ml) from Peak I following Sephacel anion exchange chromatography at pH 7.2 was incubated at room temperature with 0.1 µg/µl trypsin. After each time interval, 200 µl was removed and placed in a microcentrifuge tube with 50 µg soybean trypsin inhibitor. (a) Phosphatase activity was assayed in the absence ( ) or presence ( ) of 1 µM Ca^{2+} using histone H-1 as substrate. (b) Phosphatase activity was assayed in the absence ( ) or presence ( ) of 1 µM Ca^{2+} using phosphorylase a as substrate.


(A)
(B)
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.5
0.0
0
40
80
120
160
200
pmol ^{32}P released/min
Time (minutes)

PART II

CYCLIC AMP-DEPENDENT PROTEIN KINASE STIMULATES PROTEIN PHOSPHATASE ACTIVITY IN TICK SALIVARY GLANDS OF THE LONE STAR TICK *AMBLYOMMA AMERICANUM* (L.)

ABSTRACT

Protein phosphatase activity in the 900 x g pellet fraction of tick salivary glands was activated equally by cAMP or ATP. The inhibitor protein of the cAMP dependent protein kinase (1.25 µg/µl) inhibited protein phosphatase activity 60% while 1 µM Ca^{2+} inhibited activity 75%. Activation of protein phosphatase, partially purified on a calmodulin agarose affinity column, by cAMP, Mg • ATP and cAMP dependent protein kinase was dependent on enzyme concentration. Dilute enzyme (17 ng protein/assay) was stimulated 6-fold more than concentrated preparations (133 ng protein/assay) by these modulators, while dilute enzyme was stimulated 7-fold more than concentrated enzyme in the presence of the same modulators plus an unknown factor present in a fraction containing free regulatory subunits of cAMP-dependent protein kinase isolated by sucrose density gradient centrifugation.

cAMP caused increased ^{32}P incorporation into several endogenous proteins but also stimulated the dephosphorylation of others supporting the hypothesis that cAMP-dependent protein kinase stimulates protein phosphatase activity. Despite the ability of protein kinase inhibitor protein to inhibit phosphatase activity with exogenous phosphoprotein substrates the dephosphorylation of

endogenous proteins in response to cAMP was not inhibited by the cAMP dependent protein kinase inhibitor protein but phosphorylation of proteins in response to cAMP was inhibited. In addition, 45 and 55 KD proteins were phosphorylated in the presence of 1 μM Ca^{2+} in the absence of cAMP. The 45 KD protein was dephosphorylated in the presence of 10^{-5} M cAMP while the 55 KD protein incorporated slightly less ^{32}P.

Immunoblot analysis demonstrated that calmodulin or a calmodulin-like protein was present in fractions containing protein phosphatase activity following both anion exchange and gel filtration chromatography supporting earlier findings of an ability of μM Ca^{2+} to inhibit protein phosphatase activity. Further, protein phosphatase activity against phosphorylase a in crude preparations was inhibited 33% by a polyclonal antibody to bovine calmodulin however, activity against histone H-1 was stimulated 35% in similar experiments. The results indicate dual control of tick salivary gland protein phosphatase activity through inhibition by Ca^{2+} and activation by cAMP. Results also indicate considerable substrate specificity as to protein phosphatase and modulator interactions.

INTRODUCTION

The salivary glands of ixodid ticks and secrete numerous substances to facilitate tick feeding and are the primary organs of osmoregulation (Fawcett et al. 1986). After ingestion, the blood meal is concentrated and excess water and ions are moved across the gut epithelium to the hemocoel and returned to the host via the salivary glands. The salivary glands are under neural control and dopamine appears to be a neurotransmitter at the neuroeffector junction (Kaufman, 1976). Dopamine binds to a receptor activating an adenylate cyclase (Schmidt et al., 1981, 1982) to increase cAMP within cells of the salivary glands (Hume et al., 1984). Cyclic AMP then activates a protein kinase to catalyse the phosphorylation of proteins in the presence of Mg • ATP (Mane et al., 1985). Protein kinase consists of two cAMP receptors (R) and two active catalytic subunits (C) in most cells (Gill and Garren, 1971). However, in tick salivary glands, the holoenzymes occur in various molecular weight forms varying from M_r = 83KD to 270KD. In addition, the glands contain excess cAMP receptors (R) not associated with kinase activity (Mane et al., 1987). Cyclic AMP binds to the regulatory subunit of the holoenzyme causing dissociation of the active catalytic subunits which catalyses the phosphorylation of proteins. Protein

phosphorylation in some cells can be regulated indirectly by cAMP since several cAMP independent protein kinases are substrates of the cAMP dependent enzyme (Kiss and Steinberg, 1985). In eucaryotes the only known mode of action of cAMP is to activate cAMP dependant protein kinase (Krebs and Beavo 1979). However, recently Kiss and Steinberg (1985) and Kiss et al. (1986) have reported apparent cAMP dependent dephosphorylation of endogenous proteins.

Previous studies in this laboratory demonstrated that many proteins in tick salivary glands incorporate ^{32}P when broken cell preparations are incubated with [γ-^{32}P] ATP or when whole glands are incubated with $^{32}P_i$ (McSwain et al. 1985, 1987). However, incubation of the 900 x g pellet and the 100,000 x g pellet and supernatant fractions with cAMP and ATP caused both the phosphorylation and dephosphorylation of several proteins (McSwain Ph.D. Thesis). Because of these novel effects of cAMP, we examined the effect of cAMP and ATP on protein phosphatase activity in the 900 x g pellet. In addition, because protein phosphatase activity in tick salivary glands can be inhibited by micromolar Ca^{2+} (Williams et al., 1987), immunoblot experiments with an antibody to calmodulin were performed to determine if calmodulin or a calmodulin-like protein may be an integral subunit of the enzyme. The susceptibility of protein phosphatase activity to inhibition by the calmodulin antibody was also tested in crude enzyme preparations. We further purified the enzyme on a

calmodulin agarose affinity column and assayed activity to see if the Ca^{2+} sensitive enzyme could be activated by cAMP-dependent kinase in the presence of an apparent modulator phosphoprotein.

MATERIALS AND METHODS

Preparation of Tick Salivary Gland Homogenates

Salivary glands from feeding female lone star ticks (Amblyomma americanum (L.)) were dissected, stored and homogenized as previously described (Williams et al. 1987).

Preparation of ^{32}P Labeled Substrates

Histone H-1 was prepared from calf thymus (Jones, 1964) while phosphorylase kinase, phosphorylase b, cAMP dependent protein kinase and the inhibitor protein of the catalytic subunit of cAMP dependent protein kinase (PKI) were purchased from Sigma Chemical Co. (St. Louis). Sheep anti bovine calmodulin was purchased from Calbiochem, while biotinylated rabbit anti sheep IgG was purchased from Enzo Biochem, and streptavidin alkaline phosphatase was purchased from Bethesda Research Laboratories (BRL). [γ-^{32}P] ATP (6000 μCi/mmol) was synthesized according to Walseth and Johnson (1979). Phosphorylase b and histone H-1 were phosphorylated as previously described (Williams et al. 1987).

Assay of Protein Phosphatase Activity

Enzyme activity was assayed at 37°C by measuring the release of ^{32}P from phosphoprotein substrates (Ingebritsen and Cohen, 1983). Phosphatase activity was assayed in 50 mM PIPES buffer (piperazine N,N bis (2-ethane sulfonic acid)) pH 7.0, which contained 1 mM 2-mercaptoethanol, 20 mM EGTA, and 5 mM glycerol-2-phosphate in a final volume of 50µl. In experiments testing the effects of phosphorylation on enzyme activity, phosphoprotein substrate was preincubated 30 min in buffer prior to initiating the reaction with enzyme. The reaction was terminated with 200 µl 50% TCA and placed on ice for 10 min. 200 µl of bovine serum albumin (BSA) (30 mg/ml) was added to the mixture, the suspension was vortexed and placed on ice an additional 10 minutes prior to centrifugation at 15000 x g for 5 min. A 100 µl aliquot of supernatant was placed in 5 ml of Biocount scintillation fluid and radioactivity measured with a Beckman LS-3133T liquid scintillation counter.

The assays of phosphatase activity in fractions after both anion exchange and gel filtration chromatography were conducted as described by Williams et al. (1987). Briefly, enzyme was preincubated in buffer for 2 min prior to initiating the reaction with phosphoprotein substrate. The reaction was terminated and activity measured as previously described.

Sucrose Density Gradient Centrifugation

Density gradient centrifugation in 5-20% sucrose was preformed using a modification of the procedure described by Mane et al. (1985). Linear sucrose gradients (10.5 ml) were established in 50 mM PIPES buffer, pH 7.0 containing 10 mM EGTA. Gradients were held at 4°C for 2 hrs prior to layering the 100,000 x g supernatant on top of the gradient. Gradients were centrifuged at 105,000 x g for 40 hrs at 4°C in a Beckman SW 41 rotor. Fractions (0.65 ml) were collected from the top using an ISCO gradient fractionator. Fractions were assayed for cAMP-dependent protein kinase activity as described by Mane et al. (1985), and for phosphatase activity as previously described by Williams et al. (1987) (data not shown). Free cyclic AMP dependent protein kinase regulatory subunit activity was determined in a competitive assay using protein kinase holoenzyme from fraction 13 of the sucrose density gradient as the source of enzyme. The protein kinase assay of Mane et al. (1985) was utilized using 0.2 µM cAMP in the presence or absence of 10 µl aliquots from each fraction.

Calmodulin Agarose Affinity Chromatography

Salivary glands from 200 feeding adult female ticks were dissected as previously described (Williams et al. 1987), washed 3 times in 50 mM Tris buffer, pH 7.5, containing 1 mM 2-mercaptoethanol prior to homogenization in an identical buffer. The calmodulin agarose affinity column

(0.5 x 7.5 cm) was equilibrated at room temperature with 10 bed volumes of homogenization buffer containing 0.1 mM $CaCl_2$. The 15000 x g supernatant was made 5 mM $CaCl_2$ immediately prior to passing it over the column and unbound protein was removed with equilibration buffer. The column was washed with equilibration buffer containing 0.2 M NaCl followed by an additional wash with equilibration buffer. Protein was eluted from the column with one bed volume of buffer containing 1 mM EGTA in place of $CaCl_2$. The column was allowed to equilibrate with this buffer for 4 hrs prior to collecting 2.1 ml fractions. The eluted fractions were pooled, concentrated and used as the source of partially purified enzyme in phosphorylation experiments.

DEAE Sephacel Chromatography

Salivary glands from 500 feeding adult female ticks were dissected as previously described (Williams et al. 1987), washed 3 times in 5 volumes of 50 mM Tris buffer pH 7.5 containing 10 mM EGTA and 5 mM 2-mercaptoethanol prior to homogenization in identical buffer. The DEAE Sephacel column (1.6 x 12 cm) was equilibrated with 6 bed volumes of homogenization buffer prior to addition of protein. The column was washed with equilibration buffer and protein then eluted (1.76 ml/fraction) with a linear gradient of 0 - 0.5 M NaCl (100 ml total volume). Protein phosphatase activity was assayed as previously described.

Sephacryl S-300 Gel Filtration

Fractions following DEAE Sephacel chromatography exhibiting protein phosphatase activity were pooled (Fig. 2) concentrated on Centricon C-10 micro concentrator tubes, and dialyzed against gel filtration equilibration buffer containing 50mM MOPS, pH 6.5, 10 mM EGTA and 0.1% 2-mercaptoethanol. The sample was loaded on the Sephacryl S-300 column (0.5 x 80 cm) and 1.5 ml fractions were collected. Fractions after both anion exchange and gel filtration were assayed for protein phosphatase activity and screened by an Immunoblot procedure using sheep antibovine calmodulin (Calbiochem).

Effect of Ca^{2+} and cAMP on Dephosphorylation of Endogenous Proteins

Salivary glands from 50 feeding female ticks were sonicated twice with a Fischer Sonic Dismembrator at 6 kilocycles sec^{-1} for 20 sec. The homogenate was placed on ice for 2 min after each sonication and then centrifuged at 15000 x g for 10 min in an Eppendorf 5412 centrifuge. The supernatant was decanted and 5 µl aliquots (45 µg protein) were used in labeling experiments. The effect of cAMP and variable Ca^{2+} concentrations on the phosphorylation state of endogenous proteins was determined in the presence and absence of 10^{-5}M cAMP and 0.5 mM isobutyl methyl xanthine (IBMX) (Fig. 1). Levels of free Ca^{2+} were set with a Ca^{2+}-EGTA buffer whose concentrations were calculated using a

computer program (Perrin and Sayce, 1967). The reaction was initiated with 5 µM [$-^{32}P$] ATP (8 Ci/mmol) in a final volume of 50µl. The reaction was terminated by adding 60 µl of sodium dodecyl sulfate (SDS) stop solution containing 0.0625 M Tris-HCl, 5% 2-mercaptoethanol, 10% (w/v) glycerol, 2.3% SDS and 0.001% bromphenol blue. Samples were heated in a boiling water bath for 5 min and then electrophoresed according to the method of King and Laemmli (1971) on 10% acrylamide gels. Gels were fixed and stained in Coomassie brilliant blue, destained in 38% ethanol and 3% acetic acid and dried on a Bio-Rad slab gel drier. Autoradiographs were produced by exposing dried gels to Kodak X-OMat AR X-ray film at room temperature for periods up to 5 days.

Effect of cAMP, Mg • ATP and cAMP-dependent Protein Kinase on Protein Phosphatase Activity

Further experiments with partially purified enzyme were conducted in which either enzyme or substrate was preincubated 30 min. Preincubations containing protein kinase were stopped by adding PKI. The assay was initiated by addition of the component omitted from the preincubation. Activity was assayed as described under the assay of protein phosphatase activity.

Immunoblot Analysis and the Effect of the Calmodulin Antibody on Protein Phosphatase Activity

Aliquots (10 µl) from anion exchange and gel filtration fractions were blotted on nitrocellulose previously wetted

in 0.1 M Tris buffer pH 7.5 containing 0.1M NaCl (buffer 1) and allowed to air dry. Nitrocellulose was placed in blocking solution (buffer 1 plus 3% BSA) for 2 hrs, followed by 3 washes in buffer 1 to remove excess BSA. The nitrocellulose was immersed in primary antibody solution (Buffer 1 plus 1% BSA and 1:51,000 dilution of sheep antibovine calmodulin) for 1.5 hr with slight agitation. Unbound antibody was removed by three 5 min washes in 100 ml buffer 1. The nitrocellulose filter was placed in the secondary antibody solution (Buffer 1 plus 1% BSA and 1:10,000 dilution of biotinylated rabbit-antisheep IgG) for 1 hr and then washed 3 times in buffer 1. The filter was incubated in 10 ml of streptavidin-alkaline phosphatase solution and gently agitated for 10 min and washed twice with buffer 1. The nitrocellulose filter was finally transfered to 15 ml Buffer 3 (0.1M Tris buffer pH 9.5, 0.1M NaCl and 50 mM $MgCl_2$, 66µl nitroblue tetrazolium and 50µl 5-bromo 4-chloro-3-indolyl phosphate). The filter was incubated for 0.5 hr and the reaction terminated by transfering the filter to 20 mM Tris buffer pH 7.5 containing 0.5 mM EDTA.

Effect of Calmodulin Antibody on Protein Phosphatase Activity

Sheep anti-bovine calmodulin (Calbiochem) was reconstituted with 1 ml distilled water. The effect of this antibody on protein phosphatase activity was determined by including increasing quantities (5, 10, 15, and 20 µl) in

the phosphatase assay using either phosphorylase a or histone H-1 as substrate. Enzyme activity was determined as previously described.

RESULTS

Protein phosphatase activity in the 900 x g pellet of tick salivary glands was activated by the addition of cAMP or ATP. Activity in the presence of both cAMP and ATP was equal to that with ATP alone and was inhibited 75% by 1 μM Ca^{2+}, while PKI inhibited phosphatase activity 60% (Table 1).

The specific activity of enzyme partially purified on calmodulin agarose affinity column was 7-fold higher than basal levels of enzyme activity from the 900 x g pellet (Table I). Cyclic AMP, ATP and protein kinase added individually had little effect on protein phosphatase activity but together markedly increased activity especially at low enzyme concentrations. Previous results indicated that enzyme activity was increased by dilution (Williams et al. 1987). Here, dilute preparations (17 ng protein/assay) were stimulated by cAMP-dependent protein kinase 6-fold over that in undiluted preparations (133 ng protein/assay), while activity in the absence of cAMP-dependent protein kinase was unaffected. Addition of a fraction after sucrose density gradient centrifugation containing free cAMP-dependent protein kinase regulatory subunit increased activity 7-fold

over that in undiluted fractions in the presence of cAMP-dependent protein kinase.

To see if phosphorylation stimulated by cAMP affected enzyme activity directly or through a modulator protein phosphoprotein substrate was preincubated in the presence of cAMP, Mg • ATP and cAMP-dependent protein kinase and compared to activity where enzyme was preincubated and the reaction was initiated with substrate. Activity was 2-fold greater in the former, suggesting that phosphorylating conditions caused an increase in phosphatase activity (Data not shown). Furthermore, activity was increased and additional 60% by adding protein from sucrose density gradient fractions containing partially purified regulatory subunit in the presence of cAMP, Mg • ATP and cAMP-dependent protein kinase over that without this fraction. The results suggest that activation is caused by a modulator protein since the regulatory subunit fraction did not contain protein phosphotase activity (data not shown).

Intracellular effects of Ca^{2+} are commonly mediated by a Ca^{2+} binding protein such as calmodulin. Protein phosphatase in tick salivary glands is sensitive to physiological concentrations of Ca^{2+} even after Sephacryl S-300 gel filtration (Williams et al. 1987). Therefore, the enzyme was screened for possible presence of calmodulin with a calmodulin antibody.

Immunoblot analysis of fractions from both DEAE Sephacel (Fig. 1) and Sephacryl S-300 (Fig. 2) gel

filtration tested positive but not strongly for calmodulin with the calmodulin antibody in fractions containing phosphatase activity. A good positive control was not possible because native calmodulin did not readily bind nitrocellulose filters probably due to its acidic nature (pI 3.9). To confirm these results the ability of the calmodulin antibody to inhibit protein phosphatase activity was tested. The antibody to calmodulin inhibited phosphatase activity up to 33% with phosphorylase a as substrate but not histone H-1. With histone H-1 as substrate, antibody stimulated protein phosphatase activity 35%, calmodulin alone had no effect, while antibody plus calmodulin inhibited activity 15% (Fig. 4). These latter effects were Ca^{2+}-dependent since similar experiments in the presence of EGTA showed no effect on activity.

Because of these and previous results showing that physiological concentrations of Ca^{2+} inhibited activity (Williams et al. 1987) and the novel effects of cAMP on activating protein phosphatase activity, cAMP and Ca^{2+} were tested for their ability to differentially affect phosphorylation of endogenous proteins. Crude homogenates (15,000 x g) of tick salivary glands were incubated with 5 μM $AT^{32}P$, in the presence or absence of cAMP, PKI and 0.1 or 1.0 μM Ca^{2+} for 5 min, electrophoresed and autoradiograms produced. Cyclic AMP (10 μM) increased incorporation of ^{32}P in 140, 75, 68, 62 and 38 KD proteins (Fig. 3 lanes 1 and 2). However, cAMP also stimulated the dephosphorylation

of 47 and 51 KD proteins. PKI did not inhibit the dephosphorylation of the 47 and 51 KD proteins, but did inhibit an amount of ^{32}P incorporation into all bands (lanes 3 and 4) whose incorporated was stimulated by cAMP (compare lanes 4 and 2, Fig. 3). 0.1 μM Ca^{2+} in the absence of added cAMP caused increased ^{32}P incorporation into 38 and 75 KD proteins (lane 5) as compared to controls (lane 1, Fig. 3) but at 1 μM Ca^{2+} (lane 7) 45 and 55 KD proteins were heavily phosphorylated over controls as well (compare to lane 1). At 0.1 μM Ca^{2+}, cAMP stimulated the dephosphorylation of all but the 62 KD protein (lane 6) while at 1 μM Ca^{2+}, cAMP caused little dephosphorylation of most proteins (lane 8), and much less than 0.1 μM Ca^{2+} (compare lane 8 to lane 6).

DISCUSSION

Activation of protein phosphatase activity by cAMP and ATP in the 900 x g pellet fraction suggests that activation of the enzyme may occur through phosphorylation of a modulator protein by cAMP-dependent protein kinase. The 900 x g pellet fraction of tick salivary glands contains mitochondria and plasma membranes (McSwain et al., 1987) and 20% of total salivary gland cAMP-dependent protein kinase activity is localized in the mitochondrial fraction (Mane et al., 1985). cAMP or ATP should stimulate protein phosphatase activity by this mechanism in this fraction, if enzyme, some ATP, cAMP and modulator protein are all present. In support of this hypothesis, activity was

stimulated by both cAMP and ATP and activity was not greater than that stimulated by either cAMP or ATP alone. In addition, when enzyme was preincubated under phosphorylating conditions (i.e. in the presence of cAMP, Mg • ATP and cAMP-dependent protein kinase) phosphatase activity was lower than when substrate was preincubated under phosphorylating conditions. These results suggest that a modulator phosphoprotein responsible for activating the enzyme was dephosphorylated when preincubated with enzyme. Further evidence of a modulator phosphoprotein activated by cAMP was 60% inhibition of enzyme activity in the presence of cAMP, ATP and PKI. The fact that enzyme activity could be inhibited substantially (60 and 75%) by both PKI and Ca^{2+} suggests that the same enzyme may be inhibited by both modulators.

Enzyme after calmodulin agarose affinity chromatography was stimulated by cAMP-dependent phosphorylation, but the extent of activation was dependent on enzyme concentration. We demonstrated previously (Williams et al. 1987) that phosphatase activity after dilution was accompanied by a shift in M_r from 154 to 74 KD and activity was increased 50 fold. Interestingly, basal activity was only slightly affected by dilution here but enzyme activity stimulated by phosphorylation was markedly higher. The protein concentration in the calmodulin agarose fraction was less than half that tested in the most dilute preparation by Williams et al. (1987), suggesting that either stimulation

through dilution of basal activity was maximal or that modulators responsible for enzyme activation were absent from the partially purified enzyme source. However, it is unclear why additional dilution enhanced the ability of cAMP-dependent phosphorylation to stimulate phosphatase activity.

Protein phosphatase activity in dilute enzyme preparations, stimulated by cAMP-dependent phosphorylation, increased significantly ($P < 0.05$, Students T-test) if incubated with an aliquot containing partially purified regulatory subunit. Khatra et al. (1985) and Jurgensen et al. (1985) reported that the regulatory subunit of the cAMP-dependent protein kinase when bound to cAMP inhibited protein phosphatase activity in rabbit skeletal muscle. Since the fraction containing the regulatory subunit was not homogeneous we cannot say with certainty if R or some other protein(s) is responsible for causing the activation of protein phosphatase. However, it appears that a protein in this fraction serves in some way as a modulator in the presence of cAMP, Mg • ATP and cAMP-dependent protein kinase since the fraction alone is without protein phosphatase activity.

Labelling of endogenous proteins with [$-^{32}P$] ATP resulted in incorporation of ^{32}P into many proteins. cAMP stimulated additional incorporation of ^{32}P into several proteins, but also stimulated the dephosphorylation of 47 and 51 KD proteins (Fig. 3). Including protein kinase inhibitor along with cAMP in similar assays did not affect

the dephosphorylation of the 47 and 51 KD proteins, but did inhibit ^{32}P incorporation in all bands incorporating increased ^{32}P in response to $10^{-5}M$ cAMP alone (Fig. 3). Apparently the dephosphorylation of the 47 and 51 KD proteins requires only a small degree of phosphorylation. However, with exogenous substrates, PKI inhibited protein phosphatase activity 60% below basal levels in the 900 x g pellet fraction. Calmodulin agarose affinity purified protein phosphatase activity was also inhibited by PKI. It is interesting to note that Mane et al. (in prep.) reported that tick salivary glands contain cAMP binding proteins with molecular weights similar to those that are dephosphorylated in response to $10^{-5}M$ cAMP. Rymond and Hofmann (1982) found that phosphorylation of type II regulatory subunit increased its affinity for cAMP. It may be that phosphorylated regulatory subunit is a better substrate for protein phosphatase, and that the Km for phosphatase activity against the 47 and 51 KD phosphoproteins is much lower than that with other phosphoprotein substrates. In this model, cAMP would bind to phosphorylated regulatory subunit causing the release of the catalytic subunits and subsequent phosphorylation of an unidentified modulator protein which then activates protein phosphatase activity.

Ca^{2+} either caused more or less incorporation of ^{32}P into many bands in the presence and absence of cAMP. 0.1 µM Ca^{2+} increased ^{32}P incorporation in 38 and 75 KD proteins while 1 µM Ca^{2+} increased ^{32}P incorporation in 45 and 55 KD

proteins in addition to the 38 and 75 KD proteins. Cyclic AMP, with 0.1 μM Ca^{2+}, stimulated the dephosphorylation of all proteins, while 1 μM Ca^{2+} in the presence of cAMP resulted in slight dephosphorylation of all proteins, but the extent of phosphorylation of all but the 62 KD protein was much greater than at the lower Ca^{2+} concentrations. These results are consistent with the reported Ca^{2+}-dependent inhibition of phosphatase activity reported by Williams et al. (1987). However, the conditions of the labeling experiments make it impossible to rule out Ca^{2+}-dependent kinase activity i.e. Ca^{2+} calmodulin kinase or protein kinase C. The phosphorylation of the 45 and 55 KD proteins with 1 μM Ca^{2+} in the absence of cAMP suggests this possibility. The results of Fig. 3 suggest that variable levels of Ca^{2+} and cAMP catalyze key but complex events in controlling the reversible phosphorylation of proteins in tick salivary glands.

Fluctuations in Ca^{2+} concentrations affect the activity of numerous enzymes in many tissues (Cheung, 1982). The effects of Ca^{2+} are commonly mediated by calmodulin, which binds 4 mol Ca^{2+}/molecule. The Ca^{2+} · calmodulin complex usually binds with and activates an enzyme. The mechanism of Ca^{2+}-dependent inhibition of protein phosphatase activity is unknown but it is likely that the Ca^{2+} affect is mediated by a Ca^{2+} binding protein. Immunoblot analysis suggests the presence of calmodulin or a calmodulin-like protein in fractions after DEAE Sephacel chromatography containing

protein phosphatase activity. However, the immunologic reaction was not marked. This could have been due to lack of specificity of the antibody raised against mammalian calmodulin, the nature of the antigen-antibody reaction under present conditions or other unusual, but unknown, characteristics of the Ca^{2+} binding domain of the enzyme. The fact that enzyme activity was inhibited 30% using phosphorylase a, and activated 35% using histone H-1 as substrates suggests the possibility of either multiple substrate binding sites on the enzyme or multiple enzymes. Inhibition of enzyme activity using histone H-1 as substrate was observed in experiments with calmodulin and the calmodulin antibody. These results illustrate that a Ca^{2+}-dependent interaction is involved because EGTA blocked the effect of calmodulin and antibody plus calmodulin. Because both tick salivary gland protein phosphatase (Williams et al. 1987) and calmodulin elute from anion exchange resins at relatively high salt concentrations (Dedman and Kaetzel, 1983) the presence of calmodulin in fractions containing phosphatase activity may be coincidental. In fact, the peak response of calmodulin to antibody in immunoblot analysis occurred just prior to the peak of protein phosphatase activity. Because pure calmodulin does not adhere well to nitrocellulose, the relatively good reaction to calmodulin in those fractions may have been due to calmodulin binding to other proteins coeluting in the same fraction which then bound to the nitrocellulose more efficiently than

calmodulin. Immunoblot analysis of Sephacryl S-300 fractions suggests three peaks of response to the calmodulin antibody. The first and most prominent peak is in fraction 15 while the second is coincident with the peak of phosphatase activity in fraction 22 (Fig. 3). The final, low M_r, peak appears in fraction 38. The presence of calmodulin in fractions containing phophatase activity following Sephacryl S-300 gel filtration suggests that calmodulin may be an integral subunit of the enzyme since the phosphatase elutes at approximately 154 KD while the M_r of free calmodulin is between 16-20 KD (Masaracchia et al. 1986). The column was equilibrated with buffer containing 10 mM EGTA eliminating the possibility of calmodulin comigrating in a Ca^2 dependent manner.

These experiments demonstrate that the relative concentrations of both Ca^{2+} and cAMP are critical in regulating the phosphorylation state of endogenous proteins. Dual regulation of protein phosphatase activity by both Ca^{2+} and cAMP second messenger systems suggests that protein phosphatase activity may be central to regulating the cellular response to neural or hormonal stimuli in tick salivary glands.

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TABLE 1. cAMP-DEPENDENT PROTEIN KINASE ACTIVATES PROTEIN PHOSPHATASE ACTIVITY IN TICK SALIVARY GLANDS. EFFECT OF 10^{-5}M cAMP, 0.25 mM ATP, cAMP DEPENDENT PROTEIN KINASE (PK) (0.1 µg/µl), PROTEIN KINASE INHIBITOR (PKI) (1.25 µg/µl), 1 µM Ca^{2+} AND PARTIALLY PURIFIED REGULATORY SUBUNIT (R) ON PARTIALLY PURIFIED PROTEIN PHOSPHATASE.

Fraction	Assay	*Specific Activity	% Change
900 x g Pellet+	Enzyme	93.5 (76.4, 105.4)	-
	Enzyme + cAMP	118.1 (103.1, 122.7)	26.3
	Enzyme + ATP	126.6 (110.7, 135.4)	35.4
	Enzyme + cAMP + ATP	125.7 (103.6, 139.3)	34.4
	Enzyme + cAMP + ATP + Ca^{2+}	20.2 (16.1, 26.9)	-78.4
	Enzyme + cAMP + ATP + PKI	36.6 (33.1, 39.9)	-60.9
Calmodulin++ Agarose	Enzyme (133 ng protein)	672.9 (616.5, 729.2)	-
	Enzyme + cAMP + ATP + PK	761.4 (742.3, 780.4)	13.2
	Enzyme + cAMP + ATP + PK + PKI	308.5 (298.4, 318.6)	-54.2
	Enzyme + cAMP + ATP + PK + R	744.7 (705.3, 784.1)	10.7
	Enzyme (67 ng protein)	680.1 (619.7, 740.4)	-
	Enzyme + cAMP + ATP + PK	1014.3 (1035.9, 992.7)	49.1
	Enzyme + cAMP + ATP + PK + PKI	491.7 (479.5, 503.8)	-27.7
	Enzyme + cAMP + ATP + PK + R	1180 (1224.7,1136.5)	73.5
	Enzyme + cAMP + ATP + PK + R + PKI	456.8 (473.8, 439.8)	-32.8
	Enzyme (33 ng protein)	455.3 (475.7, 434.9)	-
	Enzyme + cAMP + ATP + PK	1672.9 (1613.8,1732.0)	267.4
	Enzyme + cAMP + ATP + PK + PKI	720.2 (750.1, 690.3)	58.2
	Enzyme + cAMP + ATP + PK + R	1886.5 (2047.1,1726.5)	314.3
	Enzyme + cAMP + ATP + PK + R + PKI	915.3 (1037.0, 793.5)	101.0
	Enzyme (17 ng protein)	583.5 (592.7, 574.3)	-
	Enzyme + cAMP + ATP + PK	4676.2 (3316.0,6036.4)	701.4
	Enzyme + cAMP + ATP + PK + PKI	2404.5 (1251.9,3557)	312.1
	Enzyme + cAMP + ATP + PK + R	5415.7 (3520.1,7311.3)	828.1
	Enzyme + cAMP + ATP + PK + R + PKI	2214.0 (1622.6,2805.3)	279.4

* Specific Activity = pmoles ^{32}P, released/mg protein/min

+ = Numerals are averages of three experiments performed in triplicate. Numerals in parenthesis are the range.

++ = Numerals are means of two experiments done in triplicate. Numerals in parenthesis are averages for the first and second experiment respectively.

Figure 1. DEAE Sephacel anion exchange chromatography at pH 7.5 of salivary gland homogenate from feeding female lone star ticks. 1.76 ml fraction were collected with a linear gradient of 0-0.5 M NaCl. Fractions were assayed for phosphatase activity with histone H-1 as substrate as described in Materials and Methods. 100 µl aliquots from each fraction were removed for use in Immunoblot analysis. 10 µl from each fraction was spotted on nitrocellulose and probed with the calmodulin antibody as described in Materials and Methods. Bar (—) indicates fractions pooled and concentrated for Sephacryl S-300 gel filtration chromatography.

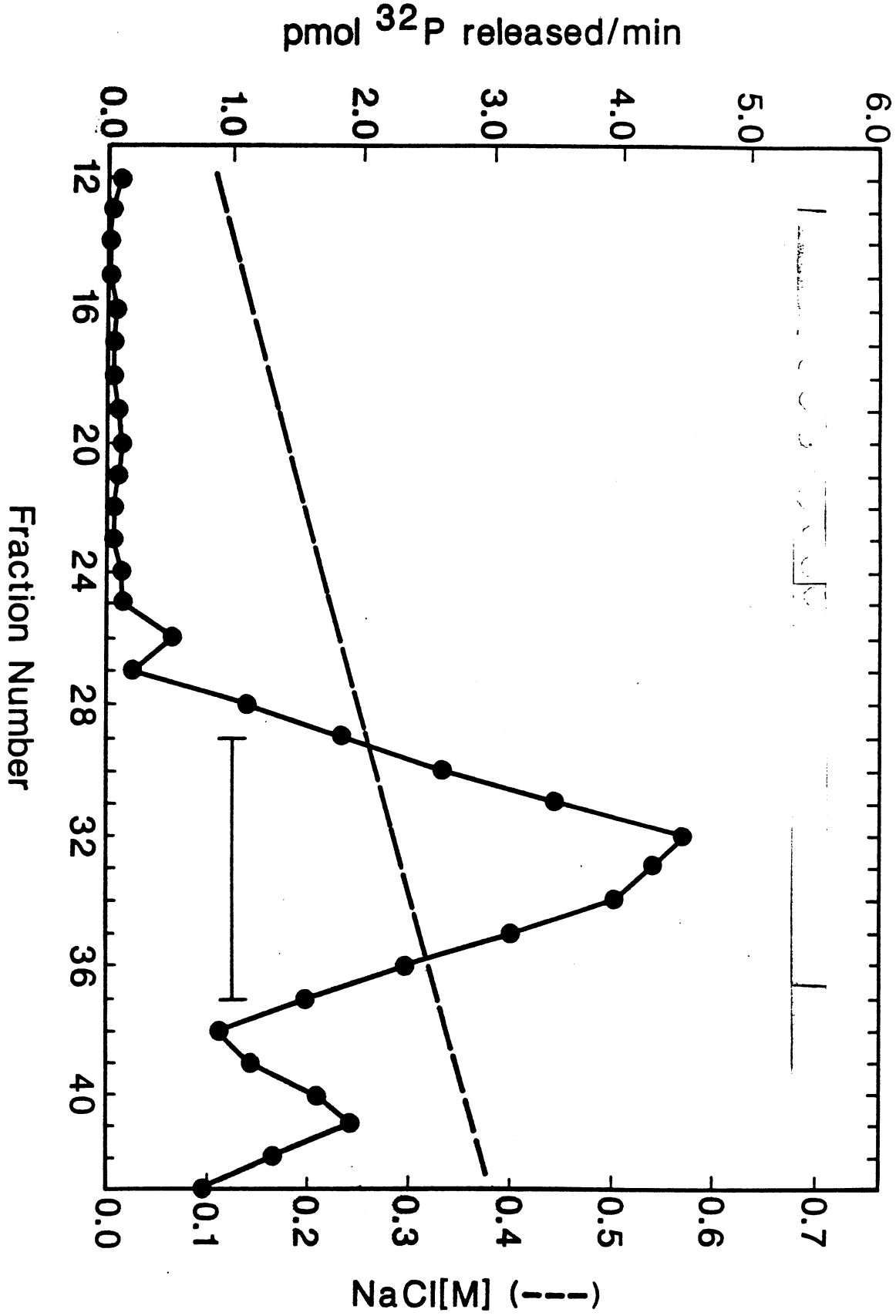

pmol 32P released/min
0.0
1.0
2.0
3.0
4.0
5.0
6.0
12
16
20
24
28
32
36
40
Fraction Number
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NaCl[M] (---)

Figure 2. Sephacryl S-300 gel filtration chromatography of enzyme from pooled and concentrated fractions from DEAE Sephacel chromatography. 1.5 ml fractions were collected and assayed for phosphatase activity using phosphorylase a as substrate as previously described. 10 µl aliquots from each fraction were blotted on nitrocellulose and probed with the calmodulin antibody as described under Materials and Methods.

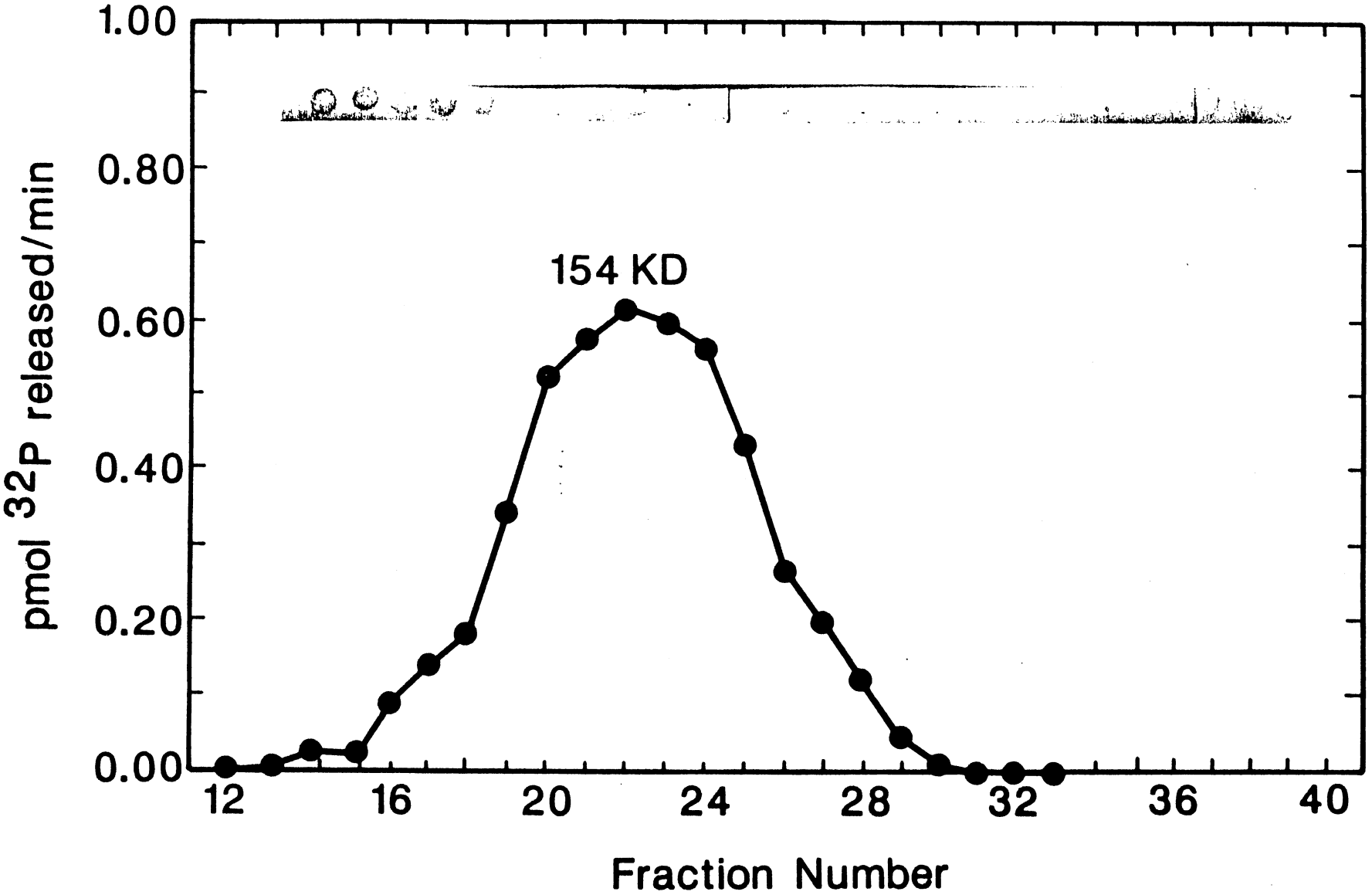

1.00
0.80
0.60
0.40
0.20
0.00
pmol 32P released/min
154 KD
12
16
20
24
28
32
36
40
Fraction Number

Figure 3. Autoradiograph showing the incorporation of ^{32}P into endogenous proteins of the 15000 x g supernatant. cAMP, Ca^{2+} and PKI were added as indicated. Arrows indicate sizes of bands whose state of phosphorylation changed in response to some treatment.

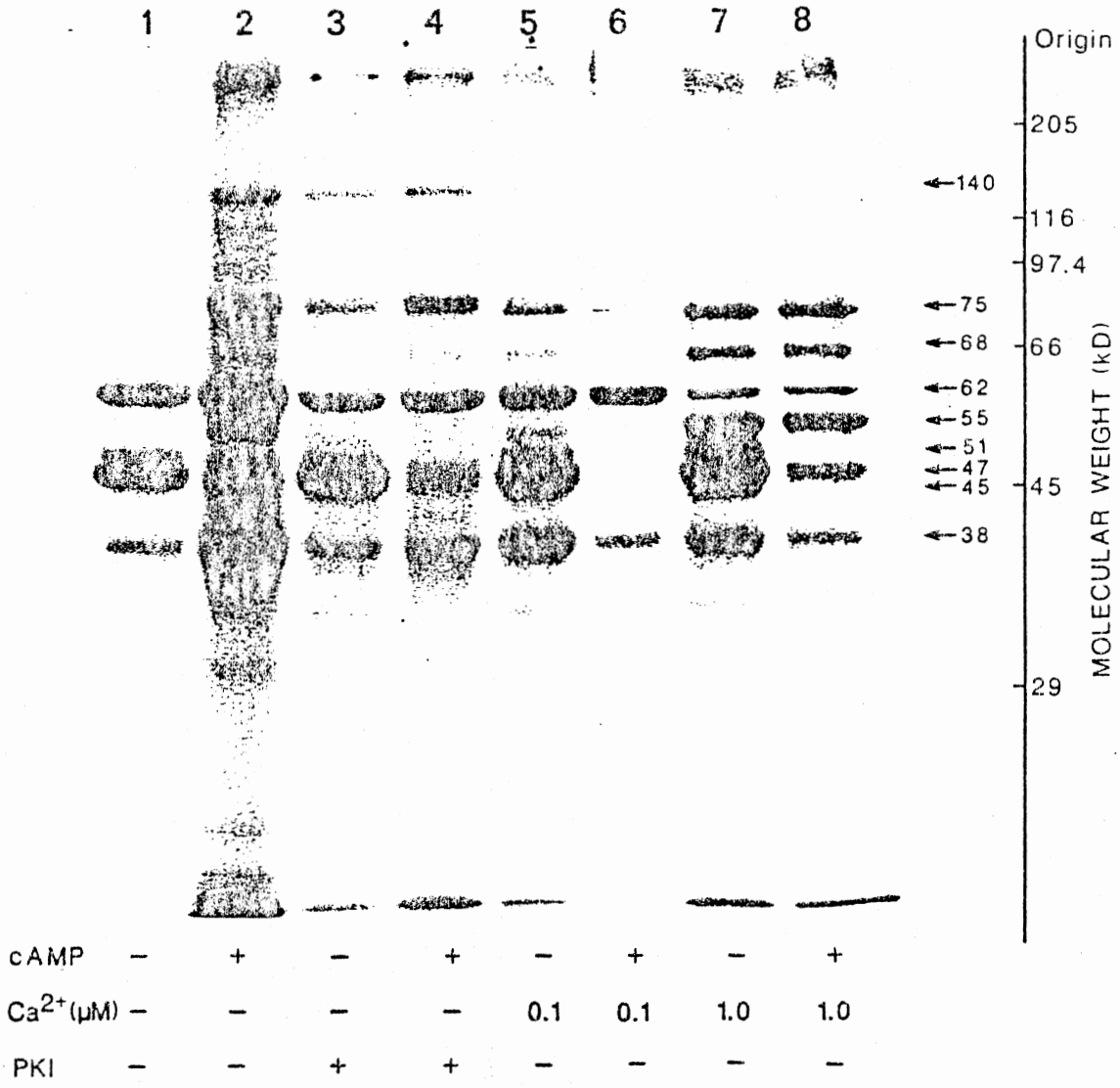
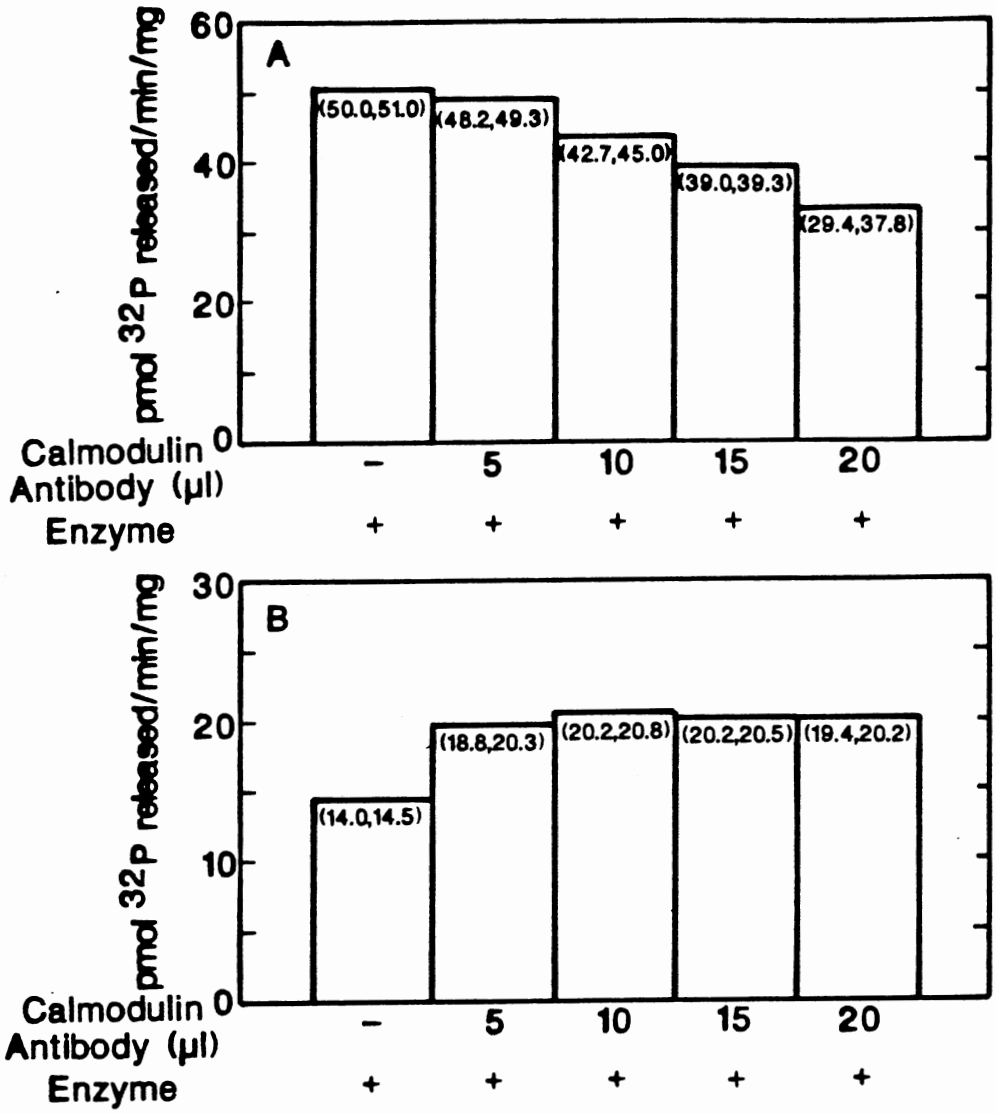

1 2 3 4 5 6 7 8
Origin
205
140
116
97.4
75
68
66
62
55
51
47
45
38
29
MOLECULAR WEIGHT (kD)
cAMP – + – + – + – +
Ca2+(μM) – – – – 0.1 0.1 1.0 1.0
PKI – – + + – – – –

Figure 4. Effect of increasing concentration of calmodulin antibody on protein phosphatase activity in the 15,000 x g supernatant. Phosphatase activity was assayed as described in Materials and Methods with 5, 10, 15 and 20 µl of the calmodulin antibody A) with phosphorylase a and B) with histone H-1 as substrate. Numerals in parenthesis are the averages for two experiments performed in triplicate.


A
pmol 32P released/min/mg
60
40
20
0
(50.0,51.0)
(48.2,49.3)
(42.7,45.0)
(39.0,39.3)
(29.4,37.8)
Calmodulin Antibody (µl)
–
5
10
15
20
Enzyme
+
+
+
+
+
B
pmol 32P released/min/mg
30
20
10
0
(14.0,14.5)
(18.8,20.3)
(20.2,20.8)
(20.2,20.5)
(19.4,20.2)
Calmodulin Antibody (µl)
–
5
10
15
20
Enzyme
+
+
+
+
+

VITA

John Philip Williams

Candidate for the Degree of
Doctor of Philosophy

Thesis: PROTEIN PHOSPHATASE ACTIVITY IN THE SALIVARY GLANDS OF THE LONE STAR TICK AMBLYOMMA AMERICANUM (L.)

Major Field: Entomology

Biographical:

Personal Data: Born in Saginaw, Michigan, October 1, 1957, the son of Douglas and Beth Williams.

Education: Graduated from Sabiaco Acadamy, Subiaco, Arkansas, May 1975; received Bachelor of Science from Oklahoma State University, May, 1981; received Master of Science from Oklahoma State University, May, 1983; completed requirements for Doctor of Philosophy at Oklahoma State University, July, 1987.

Societies and Organizations: Entomological Society of America; American Association for the Advancement of Science; Sigma Xi; Southwest Association of Parasitologists.